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13. ABSTRACT ***(Maximum 200 Words)***

A woman's lifetime risk of developing breast cancer is significantly reduced by an early first full-term pregnancy. Thus, an early first childbirth is one of the most effective naturally occurring protective events that can diminish a woman's risk of breast cancer and is a candidate for targeted chemopreventive strategies. Although there is extensive epidemiological evidence in support of parity-induced protection against breast cancer, very little is known about the molecules and pathways responsible for this protective effect. Rodent carcinogenesis models mimic the epidemiology of early parity and provide a valuable system for examining the mechanism of parity-induced protection. As a means of addressing the molecular and cellular basis of parity-induced protection, we have conducted a broad-based gene expression analysis of nulliparous and parous murine mammary glands. As a result of this analysis, we have generated a panel of genes that molecularly define the protected parous mammary gland, including differentiation markers, immune-related genes, growth factors and *TGF-β3*. To date, these findings represent the most comprehensive analysis of molecular differences induced in the mammary gland as a result of parity. Together, differential expression of distinct functional classes of molecules suggests novel mechanisms to explain parity-induced protection.

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Table of Contents

Introduction:

A woman's lifetime risk of developing breast cancer is significantly reduced by an early first full-term pregnancy. Thus, an early first childbirth is one of the most effective naturally occurring protective events that can diminish a woman's risk of breast cancer and is a candidate for targeted chemopreventive strategies. Although there is extensive epidemiological evidence in support of parity-induced protection against breast cancer, very little is known about the molecules and pathways responsible for this protective effect. Rodent carcinogenesis models mimic the epidemiology of early parity and provide a valuable system for examining the mechanism of parity-induced protection. As a means of addressing the molecular and cellular basis of parity-induced protection, we have conducted a broad-based gene expression analysis of nulliparous and parous murine mammary glands. As a result of this analysis, we have generated a panel of genes that molecularly define the protected parous mammary gland, including differentiation markers, immune-related genes, growth factors and *TGF-β3*. We identified the upregulation of several differentiation markers for mammary epithelial cells in the parous mammary gland. Additionally, we isolated genes whose expression marks the presence of a permanent population of lymphocytes and macrophages residing in the parous mammary gland. Further analysis of major categories of genes whose expression correlates with the protected parous state revealed a downregulation of multiple growth promoting molecules, such as amphiregulin, insulin-like growth factor, and pleiotrophin, concomitant with an upregulation of growth-inhibitory signaling involving *TGF-β3* and *clusterin*. To date, these findings represent the most comprehensive analysis of molecular differences induced in the mammary gland as a result of parity. Together, differential expression of distinct functional classes of molecules suggests novel mechanisms to explain parity-induced protection.

Body:

SPECIFIC AIMS:

Aim I. Identify molecular markers demonstrating parity-related changes in the rat breast.

Isolate candidate markers whose expression correlates with the protected state of the parous mammary gland using screening methods to identify differentially expressed cDNA.

Aim II. Characterize temporal and spatial expression of molecular markers in the mammary gland.

Confirm the temporal and spatial pattern of molecular marker expression in the mammary gland as a result of parity to determine the mechanism of differential regulation of candidate cDNA markers.

Aim III. Determine the expression of molecular markers with respect to parity-related reproductive histories.

Generate mammary gland tissue from animals with varying reproductive histories in order to determine parity-dependent expression of the candidate genes isolated in Specific Aim 1 and confirmed in Specific Aim 2.

TECHNICAL OBJECTIVES:

Task 1. To identify molecular markers demonstrating parity-related changes in rat breast tissue: months 1-24

- mate animals for virgin and parous breast tissue, harvest and purify total RNA: months 1-6
- generate cDNA libraries from virgin and parous rat breast tissue: months 6-12
- initiate differential screening of cDNA libraries: months 6-12
- initiate PCR-subtractive hybridization: months 6-12
- purify, sequence, and confirm expression of candidate markers: months 8-24

Using several techniques, including subtractive hybridization and microarray analysis, we have analyzed RNA from parous mice that have undergone a single round of pregnancy, lactation, and regression as well as age-matched nulliparous controls. In doing so, we have generated a panel of genes that are differentially expressed as a result of parity. Differential expression for most of these markers has been confirmed by Northern analysis on independent pools of nulliparous and parous mammary gland mRNA. By this method, we have confirmed approximately 42 molecular markers whose regulation is *consistently* altered as a result of parity. Strikingly, many of the markers identifed fall into distinct functional categories. These genes include markers of mammary epithelial differential, markers of different cellular sub-types, such as immune cells, and genes that control both cell growth and inhibition (see manuscript 1, table 1, Fig. 2).

Task 2. To characterize the temporal and spatial expression of candidate molecular markers: months 12-36

- generate parity-related developmental timepoints, prepare RNA and histological sections: months 12-18
- test temporal expression pattern of candidate markers: months 12-36
- perform *in situ* hybridization of interesting candidate markers: months 12-36

Test temporal expression pattern of candidate markers: months 12-36

In order to address the temporal pattern by which our panel of markers changes over mammary gland development, we have isolated RNA from a variety of reproductive timepoints relevant to parity. These points include virgin development (2, 5, 10 and 15 wks), pregnancy (D6, D12, and D18), lactation (D9), and involution (D2, D4 and D28).

A subset of our confirmed markers that demonstrate differential expression patterns were then subject to hybridization to Northern membranes containing mRNA from the aforementioned developmental timepoints.

We reasoned that the temporal pattern of expression of our markers would yield additional insight into the types of changes that occur as a result of parity. For example, for a gene that is upregulated as a result of parity, this increase could occur during pregnancy, lactation, involution or a combination of thereof. Gaining an understanding of the temporal pattern of expression, provides insight into the general mechanisms employed that alter expression patterns in the mammary gland of parous animals. Using this strategy, we focused on a subset of markers from the four main functional categories: differentiation, mitogenic markers, immune-related molecules, and markers of TGFβ-3 signaling.

For genes within the functional class of differentiation markers, northern hybridization demonstrated that expression of such markers was initiated throughout pregnancy, peaked during lactation, and was reduced but not eliminated throughout postlactational involution. Although the our panel of differentiation markers including, β-casein, γ-casein, ε-casein, α-lactalbumin, WAP, and WDNM1 share many similarities in expression, a careful and thorough analysis of expression throughout pregnancy, indicated that their expression patterns were not identical. In fact, monitoring the expression pattern of differentiation markers over pregnancy suggested that there are 3 distinct patterns of markers; markers of early differentiation, markers of mid-differentiation, and markers of late differentiation (see manuscript 2).

Alternatively, the expression or markers of cell growth, such as amphiregulin and pleiotrophin, were down-regulated as a result of parity and demonstrated a distinct pattern of expression over development. Steady state levels of amphiregulin and pleiotrophin mRNA are dramatically upregulated in the female mammary gland between 2 weeks and 5 weeks of age (see manuscript 1, Fig. 5b). Levels of amphiregulin and pleiotrophin expression remain relatively constant throughout nulliparous development and early pregnancy, then decrease sharply by mid-pregnancy (day 12) and remain low throughout lactation and involution (see manuscript 1, Fib. 5b). In contrast, markers of TGF-B3 signaling, or a growth inhibitory pathway, increased as a result of parity. Northern analysis of the developmental expression patterns of TGF-β3 and clusterin demonstrated that both genes exhibit maximal expression at day 2 of involution (see manuscript 1, Fig. 6b). These developmental expression profiles are consistent with a role for these molecules in cell death during mammary gland involution (Ref.). This data suggests, that the coordinate elevation inexpression of TGF-β3 along with several of its transcriptional targets suggests that the upregulation of TGF-β3 mRNA in the parous mammary gland may be accompanied by a bona fide increase in TGF-β3 activity.

Further analysis of the expression profiles of immune-related genes also demonstrated interesting temporal patterns. Northern analysis revealed that expression of the B-cell specific gene, κ-light chain (k-LC), is first detected during lactation with elevated levels of expression persisting throughout postlactational involution (see manuscript 1, Fig. 4B).

In addition, MME, a marker of macrophages, demonstrated that MME expression levels increase dramatically at day 7 of postlactational involution and remain elevated compared to age-matched nulliparous controls following 28 days of involution (manuscript 1, Fig. 4b). And finally, Northern analysis of Eta-1, which has been reported to be expressed in macrophages and T-lymphocytes as well as mammary epithelial cells, is dramatically upregulated at day 12 of pregnancy and remains high through day 7 of involution (manuscript 1, Fig. 4b). Though declining somewhat by day 28 of postlactational involution, Eta-1 expression remains markedly elevated compared to age-matched nulliparous animals.

Perform in situ hybridization of interesting candidate markers: months 12-36

In addition to furthering our understanding of the temporal pattern of expression, we performed in situ hybridization on parafin blocks of mammary gland from various developmental timepoints. By performing this analysis, we hoped to identify the cell types responsible for the expression patterns. As a result, we deteremined that the increase in expression of differentiation markers as determined by Northern analysis, was marked by an increase in epithelial cell types expressing the respective casein marker (see manuscript 2). In addition, in situ analysis of growth factors demonstrated a loss of epithelial cell sub-types expressing such markers as a result of parity (manuscript 1, Fig. 5c).

Our understanding of marker expression was further bolstered by in situ analysis by the ability to distinguish the specific cell type expressing a given marker. For the most part, our markers were expressed in the epithelial compartment of the mammary gland. Surprisingly, the cytokine, Eta-1 was also expressed the epithelium, rather than macrophages or other cell that can contribute to its expression. In contrast, markers such as kappa light chain and macrophage metalloelastase were expressed by B-cells, or macrophages and epithelium, respectively.

In summary, careful analysis of the temporal and spatial pattern of expression of our panel of markers differentially expressed as a result of parity has improved our understanding of the underlying mechanisms by which marker expression is altered. In addition, we have gained information on the cellular compartment responsible for marker expression. An extensive description of this work is appended in the manuscript "Early First Full-Term Pregnancy Results in Permanent Changes in Gene Expression in the Murine Mammary Gland".

Task 3. To generate animals of various reproductive histories and test candidate markers for their parity-dependence: months 12-36

- generate animals with the required reproductive history, prepare RNA, and histological sections: months 12-36.
- test temporal expression pattern of candidate markers: months 12-36
- perform *in situ* hybridization of interesting candidate markers: 12-36

Generate animals with the required reproductive history, prepare RNA, and histological sections: months 12-36

We have extended our analysis of parity-dependent changes to other reproductive timepoints. To address the contribution of lactation to parity-induced protection, we have generated animals that were mated "early", went through pregnancy, followed by 4 wks of postlactational regression and that had essentially no period of lactation. In addition, we have generated animals that were mated as late adults as compared to the standard "early" mating, to address the timing of the first pregnancy. We have also generated animals that went through an "early" mating, pregnancy, lactation and 26 wks of postlactational regression. In conjunction with these studies we have also generated mulitparous animals that were mated "early" but have gone through several consecutive rounds of pregnancy, followed by a standard 4 wks of regression. Nulliparous age-matched controls were also generated to match each reproductive cohort. Mammary Glands were harvested from the various nulliparous and parous animals, and mRNA and parafin-embedded blocks were generated.

Test temporal expression pattern of candidate markers: months 12-36

Interestingly, we have analyzed our panel of markers in response to these different reproductive variables and have found that many of the parity-specific changes altered after an "early" mating still hold in animals that have undergone a "late" mating, multiple matings, or a long regression. For example, parity-dependent expression patterns are still maintained in animals that have undergone 26 weeks of regression, suggesting that many parity-related changes are permanent (see manuscript 1 Fig. 7 and manuscript 2).

Perform in situ hybridization of interesting candidate markers: 12-36

We have performed extensive in situ analysis of mammary glands from virgin and parous animals following an "early" mating. At present, in situ experiments have not been performed using the newly generated paraffin-embedded mammary glands. These blocks, however, are a great resource and will be utilized in ongoing research projects in the lab.

Key Research Accomplishments:

- Identification of 41 genes that demonstrate differential expression as a result of parity in mice.

- We have demonstrated that many of the genes identified as being differentially expressed in murine tissue also demonstrate differential expression in other murine strains as well as in accepted rat models for parity-induced protection (Sprague-Dawley).

- In addition to generating a panel of markers that distinguish between a protected mammary gland, we have identified pathways that may be involved in the underlying mechanism of parity-induced protection. These pathways include mammary gland differentiation, growth-promoting signaling cascades, growth-inhibitory pathways, and a potential role for the immune system in mediating protection from cancer.

- We have provided the first molecular evidence to support the hypothesis that parity results in a more differentiated mammary gland.

- We have provided examples of various temporal patterns that can lead to differential expression patterns that occur as a result of parity.

- In addition, we have investigated the spatial pattern of expression for a subset of markers. This information was valuable in identifying the specific cell types responsible for marker expression as well as illuminating the expansion or reduction of expression in within mammary epithelial cells.

- In addition, we have provided evidence to suggest other plausible pathways that may be involved in providing protection from carcinogens.

- We have generated cohorts of animals with varying reproductive histories in order to address the impact of lactation, length of regression, and multiple births on the differential expression pattern of a subset of our markers.

Reportable Outcomes:

Publications of manuscripts supported by this grant:

Chodosh, LA, D'Cruz, CM, Gardner, HP, Ha, SI, Marquis, ST, Rajan, JV, Stairs, DB, Wang, JY, Wang, M. Mammary gland development, reproductive history, and breast cancer risk. Cancer Res. (1999) 59(7 Suppl):1765-1771s:discussion

D'Cruz, C.M., Moody, S.E., Master, S.R., Hartman, J.L, Keiper, E.A., Imeilinski, M.B., Cox, J.D., Wang, J.Y., Ha, S.I., Keister, B.A, and L. A. Chodosh. Early First Full-Term Pregnancy Results in Permanent Changes in Gene Expression in the Murine Mammary Gland (manuscript in preparation)

D'Cruz, C.M., Ha, S.I., Wang, J.Y., Marquis, S.T., and Chodosh L.A. Parity-Induced Differentiation is Dependent on Reproductive History (manuscript in preparation).

Conclusions:

We have used DNA oligonucleotides microarrays to analyze the expression of ~ 5,300 genes and ESTs in order to identify the persistent changes in gene expression in the murine mammary gland that are induced by an early first full-term pregnancy. Using this approach, we have isoloated a panel of genes whose expression is persistently altered by

a reproductive event known to reduce breast cancer risk. The expression patterns of the genes isolated reproducibly distinguish between the nulliparous and parous mammary gland in both rats and mice, as well as identify changes in the abundance of specific cell types that occur in the mammary gland as a result of parity. Our findings demonstrate at the molecular at the molecular level that parity results in a persistent increase in the differentiated state of the mammary epithelium, as has been previously suggested based on morphological criteria. In addition, our data suggest several new hypothesis for the mechanistic basis of parity-induced protection against breast cancer, including that parity may decrease the susceptibility of the mammary epithelium to malignant transformation by downregulation multiple growth promoting pathways, upregulating growth-inhibitory pathways, and /or changing the immune environment of the breast.

For the majority of genes isolated in this study, parity-induced changes in gene expression were shown to be independent of the length of postlactational involution, demonstrating that the altered patterns of expression that we have identified are persistent, if not permanent. Furthermore, we have confirmed that parity-induced changes in gene expression are largely conserved in multiple strains of mice as well as in two well-characterized rat models for parity-induced protection against breast cancer. Beyond indicating that many of the molecular changes that we have defined are conserved among rodents, our findings suggest that these changes may also be relevant to parity-induced changes in the human breast.

References:

See appendices (manuscript 1 and 2)

Early First Full-Term Pregnancy Results in Permanent Changes in Gene Expression in the Murine Mammary Gland

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ABSTRACT

Epidemiologic studies have repeatedly demonstrated that women who undergo a first full-term pregnancy early in life have a significantly reduced lifetime risk of breast cancer. Similarly, rodents that have previously undergone a full-term pregnancy are highly resistant to carcinogen-induced breast cancer compared to age-matched nulliparous controls. Despite consistent descriptions of these phenomena over the past 30 years, however, little progress has been made towards understanding the cellular and molecular basis of parity-induced protection against breast cancer. We have used high-density DNA oligonucleotide microarrays to identify a panel of differentially expressed genes that reproducibly distinguishes, in a blinded manner, between the nulliparous and parous states of the mammary gland in multiple strains of mice as well as in two widely used rat models for parity-induced protection against breast cancer. Consistent with the lifelong protective effect of parity on breast cancer risk, the changes in gene expression that we identified were undiminished after 30 weeks of postlactational involution. In addition, subsets of growth factor-related, differentiation-related, immune-related and TGF-β3-related genes were also able to distinguish in a blinded manner between nulliparous and parous mammary samples. We find that parity results in the persistent down-regulation of multiple genes encoding epithelial growth factors, such as *amphiregulin*, *pleiotrophin* and *insulin-like growth factor 1*, as well as the persistent upregulation of the growth-inhibitory molecule, *TGF-β3*, and several of its transcriptional targets. Our studies further indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as lifelong changes in the immunologic environment and hematopoietic cell types resident within the gland. These findings define a developmental state of the mammary gland that is refractory to carcinogenesis and suggest novel hypotheses for mechanisms by which parity may modulate breast cancer risk.

INTRODUCTION

Numerous epidemiologic studies have shown that women who undergo a full-term pregnancy early in life have a significantly reduced lifetime risk of breast cancer (Bain et al., 1981; Brinton et al., 1983; Ewertz et al., 1990; Layde et al., 1989; Lund, 1991; MacMahon et al., 1970; Negri et al., 1988; Paffenbarger et al., 1980; Rosner and Colditz, 1996; Rosner et al., 1994). Although other reproductive variables such as multiple full-term pregnancies and duration of lactation have also been shown to reduce breast cancer risk, these effects are generally modest and are independent of age at first childbirth (Bruzzi et al., 1988; Kvale et al., 1987; Layde et al., 1989; Paffenbarger et al., 1980; Pathak and Whittemore, 1992; Vatten and Kvinnsland, 1992; Wang et al., 1992; Yuan et al., 1988). Notably, women from different countries and ethnic groups exhibit a similar degree of parity-induced protection against breast cancer regardless of whether the regional incidence of this malignancy is high, as in Western countries, or low as in the Far East. This suggests that the reduction in breast cancer risk associated with early first full-term pregnancy does not result from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting, but rather from an intrinsic effect of parity on the biology of the breast. In principle, this protective effect could result from the pregnancy-driven terminal differentiation of a subpopulation of target cells at increased risk for carcinogenesis, from the preferential loss of target cells during postlactational involution, or from a permanent endocrine change that indirectly decreases breast cancer risk by altering either the hormonal environment or the hormonal responsiveness of cells in the mammary gland (Guzman et al., 1999; Nandi et al., 1995; Russo and Russo, 1978; Russo et al., 1982; Russo et al., 1979; Swanson et al., 1995; Thordarson et al., 1995; Thordarson et al., 2001). To date, however, little evidence exists to support any of these hypotheses at the cellular or molecular level.

Like humans, both rats and mice exhibit parity-induced protection against breast cancer. For example, administration of the carcinogens 7,12-dimethylbenz(a)anthracene or N-methylnitrosourea to nulliparous rats induces mammary adenocarcinomas that are hormone-dependent and histologically similar to human breast tumors (Dao et al., 1960; Guzman et al., 1999; Huggins et al., 1959a; Huggins et al., 1959b; Huggins et al., 1961; Moon, 1969; Moon, 1981; Russo and Russo, 1978; Russo et al., 1977; Russo et al., 1979; Sivaraman et al., 1998; Thordarson et al., 1995; Yang et al., 1999). In contrast, rats that have previously undergone a full-term pregnancy are highly resistant to the induction of breast cancer by carcinogen administration. Similar to rats, mice that have undergone an early first full-term pregnancy have also been shown to be less susceptible to carcinogen-induced mammary tumors than age-matched nulliparous controls (Medina and Smith, 1999). These studies indicate that key epidemiological features of the influence of reproductive history on breast cancer risk in humans are mirrored in rodent model systems. The use of animal models to study parity-induced protection against breast cancer is further facilitated by the many similarities in structure, function, and development that exist between the human and rodent mammary gland (Daniel and Silberstein, 1987; Russo et al., 1990). Since the mechanisms underlying parity-induced changes in breast cancer risk are likely to involve complex genetic and epigenetic events, animal models that recapitulate epidemiological findings, permit critical aspects of reproductive history to be rigorously controlled, reduce genetic variation, and permit the examination of molecular and cellular events at defined developmental stages of interest in normal tissue are critical for understanding this phenomenon.

Despite long-standing evidence for the differential susceptibility of the parous and nulliparous breast to carcinogenesis, a comprehensive analysis of the molecular and cellular

changes induced in the breast by parity has not been previously reported. Such information would not only define a protected state of the mammary gland at the molecular level, but could also provide insight into the pathways that underlie parity-induced protection. In addition, identifying a panel of molecular markers whose expression is reproducibly altered by early first full-term pregnancy would provide candidate intermediate molecular endpoints by which to monitor the efficacy of pharmacological interventions designed to mimic this naturally occurring protective event.

In this report we have used high-density oligonucleotide microarrays to analyze the impact of early first full-term pregnancy on global gene expression profiles within the murine mammary gland. This approach has led to the identification of a panel of genes whose expression in the mammary gland is persistently altered as a consequence of parity in multiple strains of mice as well as in two widely used rat models for parity-induced protection against breast cancer. Our findings demonstrate that parity induces the persistent downregulation of multiple genes encoding epithelial growth factors as well as the persistent upregulation of the growth-inhibitory molecule, *TGF-ß3*, and several of its downstream targets. In addition, our findings indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as permanent changes in the hematopoietic cell types resident within the gland. These findings provide a global molecular description of a developmental state of the mammary gland that is refractory to carcinogenesis and suggest novel hypotheses for the mechanistic basis by which parity may modulate breast cancer risk.

RESULTS

Parity Results in Permanent Morphological Changes in the Mammary Gland

In humans, parity-induced changes in breast cancer susceptibility are accompanied by morphological alterations in the mammary gland (Russo et al., 1990; Russo et al., 1992; Russo and Russo, 1993). These structural changes persist throughout life and have been interpreted as a parity-induced increase in the differentiated state of the breast (Russo et al., 1990; Russo et al., 1992; Russo and Russo, 1993). Parity-induced changes in morphology and cancer susceptibility have also been observed in the mammary glands of rats and mice (Moon, 1969; Russo and Russo, 1980; Sinha et al., 1988; Sivaraman et al., 1998; Thordarson et al., 1995; Medina and Smith, 1999). To extend these findings, we compared parity-induced changes in mammary gland morphology in Sprague-Dawley rats, a widely used model for parity-induced protection against breast cancer, with those induced by parity in C57Bl/6 and FVB mice.

Following mating at an age corresponding to the onset of puberty, rats and mice were allowed to undergo a single round of pregnancy, 21 days of lactation, and 28 days of postlactational involution. Examination of carmine-stained whole mounts prepared from these animals demonstrated that the epithelial trees of both the rat and mouse parous involuted mammary gland are more highly branched than those of age-matched nulliparous littermates (Fig. 1). Despite the marked differences in ductal branching patterns between nulliparous and parous mammary glands, hematoxylin and eosin-stained sections reveal that the relative amount of epithelium contained within the mammary fat pad is similar in both developmental states (Fig. 1 and data not shown). Thus, architectural differences between the nulliparous and parous mouse mammary gland are easily distinguishable, occur in multiple strains, are conserved among rodent species, and are analogous to those that have been described in the human breast (Russo

et al., 1990; Russo et al., 1992).

Microarray Analysis of Parity-Induced Changes in Gene Expression

Since parity-induced changes in both the structure of the mammary gland and its susceptibility to cancer are conserved between humans and rodents, we attempted to identify the molecular differences between the nulliparous and parous murine mammary gland using high density oligonucleotide microarrays. These studies were intended to define a developmentally protected state of the mammary gland at the molecular level as well as facilitate the identification of parity-induced changes in the abundance of different cell types within the mammary gland. While we anticipated that many of the differentially expressed genes identified by this approach might not play a causal role in parity-induced protection, we nevertheless considered it likely that differentially expressed genes would provide insight into parity-induced alterations in the mammary gland, including those that are responsible for the resistance of the parous gland to carcinogenesis.

Oligonucleotide microarray expression profiling was performed in triplicate on pooled mammary gland samples, each of which was derived from 15-20 animals to control for sources of biological variation such as the estrus cycle. Additionally, since stromal-epithelial interactions have been clearly shown to affect the behavior of mammary epithelial cells, expression changes were profiled in intact mammary glands with the exception that the lymph node present in the number 4 mammary gland was removed (Clarke et al., 1992; Silberstein et al., 1992; Sternlicht et al., 1999; Wiesen et al., 1999). Although the morphological changes characteristic of postlactational involution are essentially complete after 14 days (D'Cruz and Chodosh, unpublished), we chose to profile the mammary glands of parous animals after 28 days of involution to facilitate the identification of persistent changes in gene expression due to parity

rather than acute changes in gene expression due to the processes of pregnancy, lactation or involution *per se*.

Three independent, age-matched pools of total RNA derived from nulliparous (15 wk G0P0) and parous (15 wk G1P1) cohorts were hybridized to high-density oligonucleotide microarrays representing approximately 5,300 murine genes and ESTs (Mu6500). Affymetrix comparative algorithms were used to identify genes whose expression levels consistently changed as a consequence of parity. Northern hybridization analysis performed on independent nulliparous and parous mammary gland samples confirmed the differential expression of 14/14 genes identified by Affymetrix algorithms as being differentially expressed in each of the three independent microarray comparisons (Fig. 2 and data not shown). This approach, combined with Northern analysis of candidate genes identified by microarray analysis as being differentially expressed in two out of three sets of nulliparous and parous samples, resulted in the identification of 41 differentially expressed genes (Table 1 and Fig. 2). These include 10 genes that are preferentially expressed in the nulliparous mammary gland as well as 31 genes that are preferentially expressed in the parous involuted mammary gland. Notably, expression levels of *cytokeratins 5*, *8*, *14* and *18*, were not found to differ between the nulliparous and parous mammary gland, indicating that the observed changes in gene expression are not merely a consequence of the expansion or contraction of the epithelial cell compartment (Fig. 2 and data not shown).

Differentially Expressed Genes Distinguish Parous and Nulliparous Mice and Rats

Having identified a panel of genes that were differentially expressed in a parity-dependent manner in an index group of FVB mice, we asked whether the expression patterns of

these genes were sufficient to blindly distinguish nulliparous and parous mammary tissues harvested from independent groups of FVB mice, as well as from additional strains of mice. To this end, we generated 12 additional independent sets of pooled mammary gland RNA samples from parous and nulliparous FVB and Balb/c mice. These samples were subjected to high-density oligonucleotide microarray analysis on MGU74A mouse arrays and clustered based on expression profiles for the differentially expressed genes identified in our original Mu6500 array analysis. Cluster analysis performed in a blinded manner demonstrated that the expression patterns for the 41 genes identified in this study were sufficient to correctly distinguish nulliparous and parous mammary gland samples harvested from independent sets of FVB mice (Fig. 3A). Notably, the expression patterns for these same genes were also sufficient to correctly distinguish nulliparous and parous mammary gland samples harvested from Balb/c mice (Fig. 3B). These findings suggest that this panel of genes accurately and reproducibly identifies parity-induced changes in the murine mammary gland. This conclusion is further strengthened by the fact that a different generation of microarrays was used to perform these confirmatory analyses, since different oligonucleotide probe sets are used to detect these genes on the Mu6500 and MGU74A microarrays. To confirm and extend these findings, we performed Northern analysis on pools of mammary gland total RNA isolated from nulliparous and parous 129SvEv and Balb/c mice. We found that all ten genes examined in 129SvEv and Balb/c mouse strains were differentially expressed in a manner similar to that observed in FVB mice (Fig. 3F and data not shown).

Since the phenomenon of parity-induced protection against breast cancer is conserved among humans, rats, and mice, we predicted that the molecular changes that underlie this effect would be conserved across species. As Sprague-Dawley and Lewis rats represent the most

widely used models of parity-induced protection against breast cancer, we therefore wished to determine whether genes identified as having a parity-dependent pattern of expression in FVB mice would be sufficient to predict correctly the reproductive histories of rats. Accordingly, 6 independent sets of pooled mammary gland RNA samples from parous and nulliparous Lewis rats were analyzed on RGU34A high-density rat microarrays. To facilitate comparison of mouse data sets to microarray expression data obtained from rats, genes identified as being expressed in a parity-dependent manner in FVB mice were mapped via Homologene to the rat genome. Cluster analysis of rat microarray data revealed that the panel of genes identified on the basis of their parity-dependent expression in FVB mice was sufficient to correctly distinguish mammary gland samples harvested from nulliparous and parous Lewis rats (Fig. 3C). To extend these findings, we performed Northern analysis on pools of mammary gland total RNA isolated from nulliparous and parous Sprague-Dawley rats, another widely used model for parity-induced protection against breast cancer. Each of the nine genes examined in the rat exhibited a parity-dependent differential pattern of expression identical to that observed in the mouse (Fig. 3G and data not shown). Together, our findings demonstrate that the expression patterns of the genes that we have isolated are reproducibly and persistently altered as a consequence of parity and are conserved in different mouse strains and rodent species that exhibit parity-induced protection against breast cancer.

Functional Gene Categories Accurately Predict Reproductive History

Examination of the genes identified as being differentially expressed as a consequence of parity revealed several distinct functional categories (Table 1). These include: growth-promoting molecules such as *amphiregulin* (*Areg*), *pleiotrophin* (*Ptn*), and *insulin-like growth factor 1*

(*Igf1*); molecules related to epithelial differentiation such as milk proteins; molecules expressed by hematopoietic cells such as B-cells, T-cells and macrophages; and molecules involved in the TGF-*ß* pathway. In light of this observation we asked whether smaller subsets of genes representing individual functional gene categories would be sufficient to correctly distinguish nulliparous and parous mammary gland samples in a blinded manner.

To address this question, the 18 nulliparous and parous mammary samples described above were clustered based on the expression profiles for genes within each of four functional gene categories (growth-promoting; differentiation-related; immune-related; and TGF-*ß* pathway). Strikingly, even when considered in isolation, cluster analysis performed in a blinded manner demonstrated that gene expression patterns within any one of these four subgroups were sufficient to correctly distinguish parous and nulliparous mammary samples derived from FVB mice, Balb/c mice or Lewis rats (Fig. 3D, 3E and data not shown). For example, gene expression patterns for the growth factors *Areg*, *Ptn*, and *Igf1* were themselves sufficient to accurately identify all 18 mammary samples analyzed in FVB mice, Balb/c mice and Lewis rats (Fig. 3E and data not shown). Similarly, expression patterns for *TGF-ß3* and three of its downstream targets, *clusterin*, *Eta-1* and *Id-2*, were sufficient to correctly determine the reproductive histories of the animals from which these same samples were taken (Fig. 3D and data not shown). Finally, expression patterns for as few as 7 differentiation-related genes or 8 genes expressed by immune cells were sufficient to correctly distinguish parous and nulliparous samples in both mice and rats (data not shown). These findings demonstrate that expression changes within each of these four functional gene categories robustly and independently distinguish between the parous and the nulliparous states of the mammary gland in different rodent strains and species that exhibit parity-induced protection against breast cancer. As such, our findings suggest that

the downregulation of specific genes involved in epithelial proliferation, and the upregulation of genes involved in epithelial differentiation, immune regulation, and TGF-β-mediated growth inhibition represent cardinal features of parity-induced changes in the mammary gland.

Differentiation Markers are Preferentially Expressed in the Parous Mammary Gland

A prominent functional category of genes whose expression was persistently elevated in the parous involuted mammary glands of both mice and rats included markers for mammary epithelial differentiation such as *α-casein, ß-casein, γ-casein, κ-casein, whey acidic protein, lactoferrin, α-lactalbumin* and *connexin 26* (Table 1, Fig. 3 and data not shown). These observations indicate that the parity-dependent upregulation of markers for mammary epithelial differentiation is conserved among different mouse strains and rodent species that exhibit parity-induced protection against breast cancer. These findings provide the first molecular evidence supporting the hypothesis that parity results in a persistent increase in the differentiated state of the mammary epithelium (Russo et al., 1982). In addition, the preferential expression of *ADFP*, which is upregulated in differentiated adipocytes, in the parous involuted gland suggests that stromal compartments of the mammary gland may also become more differentiated as a consequence of parity (Gao et al., 2000).

Parity-Induced Changes in Hematopoietic Cells in the Mammary Gland

A second functional category of genes whose expression was elevated in the parous involuted mammary glands of both mice and rats included those that are specifically expressed in hematopoietic cells such as B-lymphocytes (*kappa light chain* and the *IgG, IgM,* and *IgA* heavy chains), T-lymphocytes (*T-cell death associated gene*), and macrophages (*macrophage*

metalloelastase and *macrophage expressed gene 1*) (Table 1, Figs. 3 and Fig. 4A). Additional genes, including *Early T-cell activation protein* (*Eta-1*), *LPS-Binding protein* and *Lipocalin-2*, that are either expressed by hematopoietic cells or are chemoattractants for these cell types were also persistently upregulated as a consequence of parity (Table 1, Figs. 3 and Fig. 4A) (Denhardt and Guo, 1993; Kjeldsen et al., 1993; Patarca et al., 1993; Schumann et al., 1990). The marked upregulation of these genes in the parous gland suggests that cells of B-lymphocyte, T-lymphocyte, and macrophage lineages may be more abundant in the parous mammary gland.

To investigate this hypothesis further, we examined the expression patterns for several immune-related genes during stages of postnatal mammary development representing puberty, pregnancy, lactation and involution. Northern analysis revealed that expression of the B-cell-specific gene, *κ-light chain* (*κ-LC*), is first detected during lactation with elevated levels of expression persisting throughout postlactational involution (Fig. 4B). The increase in *κ-LC* expression during lactation is partially masked by the dilutional effects that result from the massive increase in milk protein gene expression that occurs during this period, as evidenced by the apparent decrease in *β*-actin expression that occurs during this same period (Fig. 4B) (Marquis et al., 1995; Rajan et al., 1997). *In situ* hybridization confirmed the upregulation of *κ-LC* expression during lactation and further revealed that this upregulation is due to an increase in the number of *κ-LC* expressing cells (Fig. 4C and data not shown). The spatio-temporal pattern of *κ-LC* expression is consistent with the reported influx of lymphocytes into the breast that occurs during lactation (Asai et al., 2000; Tanneau et al., 1999; Tatarczuch et al., 2000). Surprisingly, however, our findings suggest that this lymphocyte population persists in the fully involuted gland.

Macrophage metalloelastase (*MME* or *MMP-12*) was also found to be persistently

upregulated in the mammary gland as a consequence of parity (Fig. 4A). MME is a secreted metalloprotease that cleaves plasminogen to generate angiostatin, a potent inhibitor of endothelial cell proliferation (Cornelius et al., 1998; Dong et al., 1997; Dong et al., 1998). Northern hybridization demonstrated that *MME* expression levels increase dramatically at day 7 of postlactational involution and remain elevated compared to age-matched nulliparous controls following 28 days of involution (Fig. 4B). *In situ* hybridization revealed that *MME* expression is restricted to isolated cells within the mammary stroma at day 7 of involution (Fig. 4C and data not shown). This finding is consistent with this gene's reported expression in macrophages and with previous evidence that macrophages are recruited to the breast during involution where they participate in the clearance of post-apoptotic debris (Belaaouaj et al., 1995; Strange et al., 1992). Interestingly, by day 28 of involution foci of *MME* expression became tightly associated with the epithelial compartment reflecting either a persistent population of macrophages residing within the parous epithelium or a subset of epithelial cells expressing *MME* (Fig. 4C and data not shown).

Finally, we examined the developmental basis for the persistent parity-dependent upregulation of *Eta-1* expression (Fig. 4A). Northern analysis revealed that *Eta-1* expression, which has been reported in macrophages and T-lymphocytes as well as in mammary epithelial cells, is dramatically upregulated at day 12 of pregnancy and remains high through day 7 of involution (Fig. 4B) (Ashkar et al., 2000; Tuck et al., 1999). Though declining somewhat by day 28 of postlactational involution, *Eta-1* expression remains markedly elevated compared to age-matched nulliparous animals. *In situ* hybridization analysis demonstrated that *Eta-1* expression in the parous involuted gland is restricted to a subset of mammary epithelial cells (Fig. 4C and data not shown). In aggregate, our data suggest that parity induces persistent increases in

populations of hematopoietic cells present within the mammary gland, as well as changes in cytokine expression within the mammary epithelium itself.

Parity Results in a Decrease in Growth Factor Expression

Interestingly, genes that encode growth regulatory molecules constituted more than half of genes that were found to be persistently downregulated by parity (Table 1). These include *Areg*, *Ptn*, *Igf1, leptin (Ob)*, and *thyroid stimulating hormone receptor (TshR)* (Table 1, Figs. 3 and 5A). The consistent expression patterns of these genes in FVB mice, Balb/c mice and Lewis rats indicates that their downregulation is a characteristic feature of parity-induced changes in the rodent mammary gland (Fig. 3).

We investigated the developmental expression pattern of the epidermal growth factor receptor ligand, *Areg,* and the heparin-binding mitogen, *Ptn*, to determine the basis for their preferential expression in the nulliparous gland. Strikingly, the temporal patterns of expression for these molecules are virtually identical during postnatal mammary development (Fig. 5B). Steady state levels of *Areg* and *Ptn* mRNA are dramatically upregulated in the female mammary gland between 2 weeks and 5 weeks of age, a period corresponding to the onset of ductal morphogenesis (Fig 5B) (Schroeder and Lee, 1998). Levels of *Areg* and *Ptn* expression remain relatively constant throughout the remainder of nulliparous development and early pregnancy, then decrease sharply by mid-pregnancy (day 12) and remain low throughout lactation and involution (Fig. 5B). *In situ* hybridization analysis confirmed the pregnancy-induced downregulation of *Areg* expression as well as the persistent downregulation of *Areg* expression throughout the epithelial compartment of the parous gland (Fig. 5C). Together with the observed

decreases in *Ptn* and *Igf1* expression levels, our data suggest the possibility that parity results in the downregulation of multiple pathways that stimulate epithelial proliferation.

Parity Results in Increased Mammary Expression of TGF-ß3 and its Targets

Our microarray and Northern hybridization data indicated that steady-state mRNA levels for the growth-inhibitory cytokine, *TGF-ß3,* were persistently elevated in the mammary glands of FVB mice, Balb/c mice, Lewis rats and Sprague-Dawley rats as a consequence of parity (Table 1, Figs. 3, 6, and 7). In addition, *clusterin*, *Eta-1* and *Id-2*, each of which has previously been implicated as a downstream transcriptional target of *TGF-ß3,* were also found to be persistently upregulated by parity in rats and mice (Table 1, Figs. 3, 4, 6, and 7) (Cooper and Newburger, 1998; Fagenholz et al., 2001; French et al., 1996; Jin and Howe, 1999; Shi et al., 2001; Strange et al., 1992). Northern and *in situ* hybridization analysis confirmed maximal expression of *TGF-ß3* and *clusterin* by epithelial cells at day 2 of involution as well as the persistently elevated levels of expression of these genes at day 28 of involution (Fig. 6B and 6C). These developmental expression profiles are consistent with a role for these molecules in cell death during mammary gland involution (French et al., 1996; Nguyen and Pollard, 2000; Strange et al., 1992). The coordinate elevation in expression of *TGF-ß3* along with several of its transcriptional targets suggests that the upregulation of *TGF-ß3* mRNA in the parous mammary gland may be accompanied by a *bona fide* increase in activity of the *TGF-ß3* pathway.

Parity-Induced Changes in Gene Expression Persist in the Mammary Gland

Epidemiologic observations suggest that those parity-induced changes in the mammary gland that are responsible for protection against breast cancer are likely to be permanent.

Accordingly, changes in gene expression that are involved in this protective effect would be predicted to persist for periods of involution greater than 4 weeks. To determine whether the parity-dependent changes in gene expression identified in this study persist for longer periods of involution, we analyzed cohorts of mice that were mated at 4 weeks of age, and then underwent 21 days of lactation and either 4, 16, or 30 weeks of postlactational involution. Northern analysis revealed that expression levels of *lactoferrin*, *κ-LC*, *TGF-β3*, *clusterin*, and *Eta-1*, were all consistently upregulated in the mammary glands of parous animals compared to age-matched nulliparous controls for up to 30 weeks of postlactational involution (Fig. 7). These findings indicate that for at least a subset of the genes identified in this study parity-induced changes in gene expression are essentially permanent.

DISCUSSION

The marked protection against breast cancer afforded women by an early first full-term pregnancy is a robust epidemiological phenomenon that has important clinical implications both for designing chemopreventive approaches to breast cancer and, more generally, for understanding how cancer susceptibility can be modulated by normal developmental events. We have used DNA oligonucleotide microarrays to analyze the expression of ~5,300 genes and ESTs in order to identify persistent changes in gene expression in the murine mammary gland that are induced by an early first full-term pregnancy. Using this approach, we have isolated a panel of genes whose expression is persistently altered by a reproductive event known to reduce breast cancer risk. The expression patterns of the genes isolated reproducibly distinguish between the nulliparous and parous mammary gland in both rats and mice, as well as identify changes in the abundance of specific cell types that occur in the mammary gland as a consequence of parity. Our findings demonstrate at the molecular level that parity results in a persistent increase in the differentiated state of the mammary epithelium, as has been previously suggested based on morphological criteria. In addition, our data suggest several new hypotheses for the mechanisms by which parity may modulate breast cancer risk, including that parity may decrease the susceptibility of the mammary epithelium to malignant transformation by downregulating multiple growth-promoting pathways, upregulating growth-inhibitory pathways, and/or changing the immune environment of the breast.

For the majority of genes investigated in this study, parity-induced changes in gene expression were found to be independent of the length of postlactational involution, demonstrating that the altered patterns of expression that we have identified are persistent, if not permanent. Furthermore, we have confirmed that parity-induced changes in gene expression are

conserved in three stains of mice as well as in two well-characterized rat models for parity-induced protection against breast cancer. Beyond indicating that many of the molecular changes that we have identified are conserved among rodents, our findings further suggest that similar parity-induced changes in gene expression may be seen in the human breast.

One of the most striking findings of our microarray analysis was the observation that parity induces a persistent down-regulation in the expression of multiple genes involved in the regulation of cell growth and proliferation. Specifically, the reduced expression of *Areg, Ptn, Igf1, TshR, and Ob* suggest that multiple mitogenic pathways may be downregulated in the mammary gland as a consequence of parity. Of note, elevated expression of *Areg, Ptn* and *Igf1* have each been implicated in the pathogenesis of human breast cancer (Harris et al., 1992). AREG, a ligand for the EGFR, has been shown to be overexpressed in 35-50% of primary human breast cancers (LeJeune et al., 1993; Normanno et al., 1995; Panico et al., 1996). Consistent with this, *Areg* is overexpressed in hyperplastic stages of mammary tumor development in MMTV-PyMT and MT-TGFα transgenic mice (Niemeyer et al., 1999), and is a potent stimulator of anchorage-dependent growth in nontransformed MCF-10 cells (Normanno et al., 1994). Moreover, targeted inactivation of *Areg* in mice causes a marked delay in ductal elongation during puberty and downregulation of *Areg* expression in transformed mammary epithelial cell lines results in growth inhibition and reduced tumorigenicity (De Luca et al., 1999; Luetteke et al., 1999; Ma et al., 1999). These findings indicate that Areg plays an important role in promoting mammary epithelial cell proliferation and predict that the parity-dependent downregulation of *Areg* could contribute to a decrease in the susceptibility of the parous mammary gland to cancer.

Similar to *Areg*, the heparin-binding growth factor, *pleiotrophin*, was also found to be

down-regulated in the parous mammary gland. Pleiotrophin has been implicated in angiogenesis and mammary tumor progression and a majority of primary human breast tumors display high levels of *PTN* mRNA expression, as do carcinogen-induced rat mammary tumors (Fang et al., 1992; Kurtz et al., 1998). Moreover, Ptn overexpression in NIH 3T3 cells induces transformation and confers the ability to form tumors in nude mice (Chauhan et al., 1993). Conversely, overexpression of a dominant negative PTN mutant reverses the transformed phenotype of the human breast cancer cell line MDA-MB-231 (Zhang et al., 1997). PTN has also been reported to stimulate endothelial cell growth, migration, and capillary tube formation. Furthermorer, tumors formed by injection of *PTN*-transfected MCF-7 breast carcinoma cells into mice exhibit enhanced growth, increased endothelial proliferation, and increased vascular density compared to control cells (Choudhuri et al., 1997; Papadimitriou et al., 2001; Souttou et al., 2001). As such, like *Areg*, the downregulation of *Ptn* represents a biologically plausible mechanism that could contribute to parity-induced protection against breast cancer.

Our finding that *Igf1, Ptn, Ob* and *TshR*, are downregulated by parity raises the possibility that parity-induced alterations in other growth promoting pathways may also contribute to protection against breast cancer. This hypothesis is particularly intriguing in light of recent findings that a strong positive correlation exists between circulating IGF1 concentrations and breast cancer risk among premenopausal women (Hankinson et al., 1998). Moreover, IGF1 levels are elevated in women with breast cancer, the IGF1 receptor is overexpressed and highly activated in human breast cancer cells, and expression of the IGF downstream signaling molecule, IRS-1, is correlated with decreased disease-free survival (Peyrat and Bonneterre, 1992; Pezzino et al., 1996; Pollak, 1998; Rocha et al., 1997). Significant evidence also exists for a critical interplay between IGF1R and estrogen receptor signaling in

that IGF action potentiates the mitogenic response of breast cancer cells to estrogen (Lee et al., 1997). These observations are consistent with a tumor-promoting role for IGF1 in the breast as IGF1 has both mitogenic an anti-apoptotic actions on mammary epithelial cells via activation of signaling molecules such as PI3 kinase and AKT1. This pathway is clearly implicated in human breast cancer by virtue of the increased breast cancer susceptibility observed in women bearing germline mutations in *PTEN*, a tumor suppressor gene whose product negatively regulates the AKT survival pathway (reviewed in (Fry, 2001; Petrocelli and Slingerland, 2001).

In aggregate, our studies demonstrate that parity induces a persistent down-regulation in the expression of multiple genes involved in the regulation of cell growth and proliferation. Nevertheless, while decreased epithelial proliferation rates in parous animals have been reported by some investigators other studies have failed to find a consistent difference in cellular proliferation rates between parous and nulliparous animals (Russo and Russo, 1987; Russo and Russo, 1993; Russo and Russo, 1999; Sinha et al., 1988; Sivaraman et al., 1998). Interestingly, Sivaraman and colleagues have recently shown that mimicking the protective effect of parity by treatment with estradiol and progesterone results in a block to mammary epithelial proliferation following a carcinogen challenge, and p53 has been implicated as a potential mediator of this effect (Sivaraman et al., 2001). As such, it is possible that the differential expression of *Areg*, *Ptn*, *Igf1* and other growth-promoting molecules that we have observed in this study may only manifest themselves as a difference in epithelial proliferation in the context of the response to a specific carcinogenic challenge. Further studies will therefore be required to determine the significance of these findings.

In addition to decreases in growth factor gene expression, we have also identified a parity-dependent increase in the expression of *TGF-β3* and several of its downstream

transcriptional targets in the mammary gland. The well-described role of *TGF-ß3* in growth inhibition makes the upregulation of this pathway a biologically plausible contributing factor to parity-induced protection against breast cancer. For example, overexpression of TGF-ß isoforms inhibits mammary epithelial proliferation, enhances mammary epithelial apoptosis, and can suppress tumorigenesis in the mammary gland in response to carcinogens or oncogenic stimuli (Silberstein and Daniel, 1987; Daniel et al., 1989; Nguyen and Pollard, 2000; Pierce et al., 1995). Conversely, downregulation of TGF-ß activity results in increases in epithelial proliferation as well as in spontaneous and induced tumorigenesis in the mammary gland (Bottinger et al., 1997; Gorska et al., 1998). These properties of TGF-ß isoforms suggest that this family of molecules possesses significant tumor suppressor activity in the mammary gland and may contribute to parity-induced protection against breast cancer. While our data do not directly demonstrate activation of the *TGF-ß3* signaling pathway in the parous mammary gland, the coordinate regulation of several downstream transcriptional targets of *TGF-ß3* lends support to this model. Further investigation of the activation of other downstream molecules in this pathway, such as SMAD 2, SMAD 3 and SMAD 4 would provide additional evidence for the upregulation of this pathway.

It has previously been proposed that parity-induced protection against breast cancer may be mediated by an increased state of differentiation of the parous mammary gland (Russo and Russo, 1978; Russo et al., 1982). Our data provide molecular evidence to support the contention that the epithelial compartment of the parous mammary gland is more differentiated than that of the nulliparous gland, as well as evidence to suggest that parity may also increase the differentiated state of the stromal compartment of the mammary gland. Notably, Ginger *et al.* recently reported the use of suppression subtractive hybridization-PCR to identify genes that are

persistently upregulated in the mammary glands of Wistar-Furth rats following treatment with estrogen and progesterone (Ginger et al., 2001). In addition to novel genes and genes of unknown function, several of the differentially expressed genes isolated in this study encode proteins that are markers for mammary epithelial differentiation, such as *α-casein, ß-casein,* and *κ-casein,* or are involved in regulating cellular proliferation. Nevertheless, the recent finding in rats that the dopamine antagonist, perphenazine, induces epithelial differentiation yet does not protect against carcinogen-induced mammary tumorigenesis casts doubt on a central role for differentiation in parity-induced protection against breast cancer (Guzman et al., 1999). As such, while a persistent increase in the differentiated state of the mammary gland remains a biologically plausible mechanism for reducing cancer susceptibility, this finding may nevertheless be unrelated to the mechanism by which parity reduces breast cancer risk. However, since the nature or extent of epithelial differentiation induced by perphenazine may differ from that induced by parity, further studies will be required to conclusively rule out differentiation as a contributing factor.

It should also be noted, however, that while differentiation *per se* may not protect against breast cancer, protection could be conferred by the direct action of a gene product whose expression is persistently upregulated in the differentiated gland. For example, the milk protein gene *lactoferrin*, which is upregulated ~5-fold by parity, not only represents a marker for mammary epithelial differentiation but also has been shown to suppress the proliferation of human breast cancer cells *in vitro* and tumor growth *in vivo* by inhibiting G1 cyclin-dependent kinases (Damiens et al., 1999; Hurley et al., 1994). In addition, lactoferrin is able to activate lymphokine-activated killer cells and potentiate NK cell-mediated cytotoxicity against breast cancer cell lines (Bezault et al., 1994; Damiens et al., 1998; Shau et al., 1992). Consistent with

these findings, lactoferrin has been demonstrated to inhibit the growth of transplantable tumors and to reduce experimental metastasis in mouse models (Bezault et al., 1994). These findings highlight the possibility that changes in gene expression that are related to differentiation may contribute to parity-induced protection against breast cancer by direct or indirect mechanisms.

In the course of the above experiments we were surprised to find the parity-induced upregulation of genes whose expression marks distinct classes of hematopoietic cells. These observations suggest that parity results in a persistent increase in the number of macrophages, B-lymphocytes and T-lymphocytes that reside within the mammary gland. In addition, we have shown that parity induces changes in the expression of specific cytokines in the mammary gland, at least some of which occur within the epithelial compartment. In particular, the dramatic increase in epithelial expression of the cytokine *Eta-1,* which has been shown to enhance migration of activated macrophages, to influence macrophage cytokine production, and to increase IgG and IgM expression in activated B cells, suggests a model for paracrine signaling mechanisms by which parity-induced changes in epithelial gene expression may influence the function of immune cells within the gland (Ashkar et al., 2000; Naot et al., 1997; Weber and Cantor, 1996; Zohar et al., 2000). Such paracrine signaling mechanisms could represent a route by which parity creates an environment that is refractory to tumorigenesis. More broadly, our findings suggest that parity may induce widespread changes in the immunological environment of the mammary gland that could plausibly affect cancer susceptibility either by tumor surveillance mechanisms, or by the parity-dependent creation of an environment that is less conducive to transformation, tumor establishment or tumor progression.

Consistent with the hypothesis that parity may induce changes in the mammary environment that make it less hospitable for tumor establishment and progression, we have found

that the secreted anti-angiogenesis factor, *MME*, is highly expressed by a subset of epithelial cells in the involuted parous mammary gland. Cleavage of plasminogen by MME produces angiostatin, which potently inhibits endothelial cell proliferation *in vivo* (Cornelius et al., 1998; Dong et al., 1997; Dong et al., 1998). Moreover, gene transfer of MME into murine melanoma cells inhibits angiogenesis as well as primary tumor growth (Gorrin-Rivas et al., 2000). Consistent with this *in vivo* mechanism of action, *MME* expression in human hepatocellular carcinomas correlates highly with both angiostatin protein levels and patient survival (Gorrin-Rivas et al., 1998). Thus, upregulation of *MME* expression in the parous mammary gland may suppress the neovascularization that is required for tumor growth.

Finally, the realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that naturally occurring events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that while it is now possible to identify women who are at elevated risk for developing breast cancer, few interventions currently exist. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent an attractive alternative. It is to this end that an early first full-term pregnancy has been proposed as a logical paradigm on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by our lack of understanding of the mechanisms by which reproductive events alter breast cancer risk. Understanding these mechanisms will ultimately facilitate the design of safe and effective hormonal chemoprevention regimens. Moreover, the development and testing of such regimens will be facilitated by the identification and use of intermediate molecular endpoints that accurately detect changes in the breast associated with changes in breast cancer risk. We have chosen to exploit the defined

relationship between parity and carcinogenesis in the breast to generate surrogate endpoint biomarkers for changes in the breast associated with a reduction in breast cancer risk. While our findings do not address whether the molecular and cellular alterations identified in this study are causally related to parity-induced protection against breast cancer, they do suggest promising new avenues for investigation. We believe that such biomarkers will ultimately prove essential for understanding the molecular and cellular basis of parity-induced protection against breast cancer, and for the rational design and testing of hormonal chemoprevention regimens aimed at mimicking this naturally occurring protective event.

EXPERIMENTAL PROCEDURES

Animals and Tissues

FVB, Balb/c, C57Bl/6 and 129SvEv mice and Sprague-Dawley and Lewis rats were housed under barrier conditions with a 12 hr light/dark cycle and access to food and water *ad libitum*. Parous rodents were generated by mating 4-week-old mice or 9-week-old rats. Following parturition, animals were allowed to lactate for 21 days, at which time litters were weaned. Parous animals underwent 28 days of postlactational involution prior to sacrifice, at which time the #3-5 mammary glands were harvested and snap frozen, as were those of age-matched nulliparous controls. With the exception of glands used for whole mounts, the lymph nodes within gland #4 were removed. Additional sets of mice were allowed to undergo either 16 weeks or 30 weeks of postlactational involution.

Whole Mounts and Histology

Number 4 mammary glands were mounted on glass slides, fixed overnight in neutral buffered formalin, and transferred to 70% ethanol. For whole mounts, glands were rinsed in water for 5 min and stained in a filtered solution of 0.2% carmine (Sigma) and 0.5% aluminum potassium sulfate for 1-3 days. Glands were then dehydrated sequentially through 70%, 90%, 100% ethanol for 15 min. each, then de-fatted and stored in methyl salicylate. For histological analysis, fixed glands were blocked in paraffin, sectioned, and stained with hematoxylin and eosin.

RNA Isolation and Northern Analysis

Snap-frozen tissue was homogenized in guanidine thiocyanate supplemented with 7 µl/ml 2-mercaptoethanol, and RNA isolated by centrifugation through cesium chloride as previously described (Rajan et al., 1996). Equal amounts of RNA from each of 15-20 mice or 10 rats were combined for each independent pool. Total RNA was separated on a 1% LE agarose gel, and passively transferred to Gene Screen (NEN). Northern hybridization was performed per manufacturer's instructions using PerfectHyb Plus Hybridization Buffer (Sigma) and ^{32}P-labeled cDNA probes corresponding to Genbank sequences represented on the Affymetrix oligonucleotide microarray Mu6500 Gene Chip.

Oligonucleotide Microarray Hybridization and Analysis

Approximately 40 µg of total pooled RNA from each sample was used to generate cDNA and biotinylated cRNA as described (Lockhart et al., 1996). Hybridization to a set of Affymetrix Mu6500K microarrays was performed per manufacturer's instructions. Following washing and staining with streptavidin-phycoerythrin, chips were scanned using a Hewlett-Packard Gene Array Scanner. Grid alignment and raw data generation was performed using Affymetrix GeneChip 3.1 software. Raw gene expression levels were scaled and normalized data sets were compared using Affymetrix algorithms to identify differentially expressed genes.

For clustering analysis, probe sets for differentially expressed genes identified in the Mu6500 array analysis were mapped to corresponding probe sets on Affymetrix MGU74 microarrays via the Unigene and LocusLink databases. A list of orthologous probe sets on Affymetrix RGU34 chip was generated using matches obtained by querying each differentially expressed gene identified in the mouse against the Homologene database

(http://www.ncbi.nlm.nih.gov/HomoloGene/). Of the resulting matches, only curated and calculated reciprocal best match Homologene hits were selected for further analysis. Data were scaled such that the mean signal intensity was equivalent across all array samples excluding the top and bottom 2% of data points. Samples were standardized prior to cluster analysis such that the median expression level and standard deviation for each gene equaled 0 and 1, respectively, across the set of samples being clustered. The data was then filtered to include only probe sets for genes shown to be differentially expressed between parous and nulliparous mouse samples (Table 1), or the orthologues for these genes in the rat. Cluster software was used to generate hierarchical clustering trees, which were visualized using Treeview (M. Eisen; http://www.microarrays.org/software).

In Situ Hybridization

In situ hybridization was performed as described (Marquis et al., 1995). Antisense and sense probes were sythesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing the same sequences used for Northern hybridization analysis.

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TABLE LEGENDS

Table 1. Genes that are differentially expressed in the mammary glands of parous involuted and age-matched nulliparous mice. Genes whose differential expression was confirmed by Northern analysis are indicated (Y). Genes whose differential expression was not tested (ND) by Northern hybridization, but that displayed a consistent change in expression among 3/3 microarrray experiments, were included. Clustering categories denote genes whose expression profiles were used to test the predictive power of functional gene categories to distinguish parous and nulliparous samples.

FIGURE LEGENDS

Figure 1. Parity-induced morphological changes in mice and rats. Carmine-stained whole mounts (left 6 panels) from parous involuted Sprague-Dawley rats and C57Bl/6 and FVB mice each display increased ductal branching as compared to the age-matched nulliparous gland. Magnification 25X. Histological analysis of hematoxylin and eosin-stained sections (right 2 panels) demonstrates that the proportion of epithelial cells is similar in the nulliparous and parous mammary glands. Magnification 400X.

Figure 2. Confirmation of parity-induced changes in gene expression. Northern hybridization analysis of gene expression for candidate genes identified by microarray analysis. Expression levels were determined for independent pools of mammary gland total RNA, each of which was derived from 15-20 age-matched nulliparous (lanes 1-3) or parous (lanes 4-6) animals. Genes downregulated as a consequence of parity included *superoxide dismutase III (SOD3), thyroid stimulating hormone receptor* (*Tshr*), *MUC18*, and *carbonic anhydrase isozyme III* (*CAIII*). Genes upregulated by parity included κ-casein, *adipocyte differentiation-related protein* (*ADFP*), *carboxyl ester lipase* (*CEL*), *carbonic anhydrase isozyme II* (*CAII*), and *adenosine deaminase* (*ADA*). Comparable epithelial cell content in nulliparous and parous mammary glands is demonstrated by equivalent levels of *CK18* expression when normalized to *β-actin* or 28S rRNA loading controls.

Figure 3. Differentially expressed genes identified by microarray analysis reproducibly distinguish between nulliparous and parous mammary tissues in mice and rats. (A) Six

independent FVB samples (3 parous and 3 nulliparous pooled samples; 8-10 mice per pool) were analyzed on Affymetrix MGU74A microarrays and clustered based on the expression patterns of genes identified as being differentially expressed in an independent set of parous and nulliparous FVB samples analyzed on Affymetrix Mu6500 microarrays. (B) Six independent Balb/c samples (3 parous and 3 nulliparous pooled samples; 3 mice per pool) were analyzed on Affymetrix MGU74A microarrays and clustered based on the expression patterns of genes identified as being differentially expressed in an independent set of parous and nulliparous FVB samples analyzed on Affymetrix Mu6500 microarrays. (C) Six Lewis rat samples (3 parous and 3 nulliparous pooled samples; 3-4 animals per pool) were analyzed on Affymetrix RGU34A microarrays. Genes identified as being differentially expressed in parous and nulliparous mice based on the analysis of Mu6500 microarray data were mapped via Homologene to the rat genome in order to perform clustering operations as above. (D) A subset of differentially expressed genes associated with the TGF-ß3 pathway were used to cluster the 12 murine nulliparous and parous samples described in A and B. (E) A subset of differentially expressed genes encoding epithelial growth factors were used to cluster the 6 rat samples described in C. Genes listed in Table 1 that were not represented on the MGU74A or RGU34A microarrays were omitted from the clustering analysis. F) Probes for the indicated genes were hybridized to Northern membranes containing pools of total RNA from 3 parous (P) and 3 age-matched nulliparous (N) 129SvEv and Balb/c mice G) Probes for the indicated rat genes were hybridized to Northern membranes containing pools of total RNA from 10 parous (P) and 10 nulliparous (N) Sprague-Dawley rats. *ß*-actin and 28S rRNA are shown as loading controls.

Figure 4. Differential expression of lymphoid and myeloid markers identify parity-induced changes in cell populations within the mammary gland. (A) Northern analysis of three independent pools of nulliparous (lanes 1-3) or parous (lanes 4-6) mammary gland total RNA isolated from FVB mice demonstrates parity-induced markers for macrophages (*MPEG1*), B-lymphocytes (*KLC*), and T-lymphocytes (*TDAG*). Hybridization to a probe for *ß-actin* is shown as a loading control. (B) Northern hybridization of *MME, KLC* and *Eta-1* probes to total mammary gland RNA isolated from female mice at the indicated developmental stages. Expression levels are compared to those of *ß-actin* to account for dilutional effects due to large-scale increases in milk protein gene expression during late pregnancy and lactation. (C) *In situ* hybridization analysis of *MME, KLC* and *Eta-1* expression in the mammary gland at the indicated developmental stages. Representative bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with ^{35}S-labeled *MME, KLC* and *Eta-1* specific antisense probes are shown. No signal over background was detected in sections hybridized with sense probes (data not shown). Exposure times were identical for all dark-field photomicrographs to facilitate comparison of gene expression levels. Magnification 300x.

Figure 5. Parity-induced downregulation of growth-promoting molecules in the mammary gland. (A) Northern hybridization analysis of three independent pools of nulliparous (lanes 1-3) or parous (lanes 4-6) mammary gland total RNA isolated from FVB mice demonstrates decreased expression of mitogenic signaling molecules. *ß-actin* is shown as a loading control. (B) Northern hybridization analysis of the developmental patterns of *Areg* and *Ptn* expression in the mammary gland. The 28S rRNA band is shown as a loading control. (C) *In situ*

hybridization analysis of *Areg* expression at the indicated developmental stages. Bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with an ^{35}S-labled *Areg*-specific antisense probe. No signal over background was detected in sections hybridized with a sense *Areg* probe (data not shown). Exposure times were identical for all dark-field photomicrographs to facilitate comparison of gene expression changes. Magnification 300x.

Figure 6. Parity-induced increases in *TGF-ß3* and *clusterin* expression in the mammary gland. (A) Northern analysis of three independent pools of nulliparous (lanes 1-3) and parous (lanes 4-6) mammary gland RNA isolated from FVB mice demonstrates increased expression of *TGF-ß3* and *clusterin* in the parous gland. *ß-actin* is shown as a loading control. (B) Northern hybridization analysis of the developmental patterns of *TGF-ß3* and *clusterin* expression. Hybridization to *ß-actin* is shown as a control for loading and for dilutional effects of milk protein gene expression. (C) *In situ* hybridization analysis of *TGF-ß3* and *clusterin* expression at the indicated developmental stages. Bright-field corresponding to *clusterin* analysis (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with ^{35}S-labeled *TGF-ß3* or *clusterin* antisense or sense probes. Magnification 300x.

Figure 7. Parity-induced changes in gene expression are permanent. Northern hybridization analysis for the indicated genes performed on pools of RNA from the mammary glands of parous animals that had undergone increasing periods of involution and of their age-matched nulliparous controls. Parous animals were mated at 4 wks of age, and underwent 21 days of lactation and

either 4 wks, 16 wks, or 30 wks of postlactational involution. β-actin is shown as a loading control.

Table 1: Differentially Expressed Genes

Genes Down-Regulated

Sequence Identity	Accession Number	Function	Clustering Category	Avg. Fold Change	Northern
Amphiregulin	L41352	Growth Factor	Growth-promoting	2.8	Y
Pleiotrophin	D90225	Growth Factor	Growth-promoting	2.0	Y
Insulin-Like Growth Factor 1B	W10072	Growth Factor	Growth-promoting	2.2	ND
Insulin-Like Growth Factor 1A	X04480	Growth Factor	Growth-promoting	1.6	Y
Thyroid Stimulating Hormone Receptor	U02602	Hormone Receptor	Growth-promoting	2.0	Y
Leptin	U18812	Hormone	Growth-promoting	2.0	Y
Ig Superfamily Containing Leucine-rich repeat	AA059664	Cell Adhesion		2.5	Y
MUC 18	AA088962	Cell Adhesion		2.0	Y
Superoxide Dismutase III	X84940	Oxidoreductase		2.5	Y
Carbonic Anhydrase III	M27796	Hydratase		1.4	Y

Genes Up-Regulated

Sequence Identity	Accession Number	Function	Clustering Category	Avg. Fold Change	Northern
Whey Acidic Protein	J00544	Milk Protein	Differentiation	24	Y
Gamma-Casein	D10215	Milk Protein	Differentiation	20	Y
Alpha-Lactalbumin	M80909	Milk Protein	Differentiation	13	Y
Lactoferrin	J03298	Iron Transport/Milk Protein	Differentiation	4.8	Y
Alpha-Casein	M36780	Milk Protein	Differentiation	4.2	ND
WDNM1	X93037	Protease Inhibitor/Milk Protein	Differentiation	3.5	Y
Beta-Casein	X04490	Milk Protein	Differentiation	3.3	Y
Kappa-Casein	M10114	Milk Protein	Differentiation	2.9	Y
Adipocyte Differentiation Related Protein	M93275	Differentiation	Differentiation	1.8	Y
Carboxyl Ester Lipase	U37386	Lipid Degradation	Differentiation	6.6	Y
LPS-Binding Protein	X99347	Antibacterial/Milk Protein	Differentiation	6.5	Y
Lysozyme P	M21050	Antibacterial/Milk Protein		2.0	ND
Immunoglobulin M Heavy Chain	ET61785	Immunoglobulin	Immune	30	Y
Immunoglobulin G Heavy Chain	ET61798	Immunoglobulin	Immune	13	Y
Immunoglobulin A Heavy Chain	J00475	Immunoglobulin	Immune	21	Y
Immunoglobulin Kappa Light Chain	X16678	Immunoglobulin	Immune	7.0	Y
Macrophage Metalloelastase (MMP12)	M82831	Metalloprotease	Immune	2.1	Y
Macrophage Expressed gene 1	L20315	Cell Signalling	Immune	1.7	Y
Lipocalin 2	W13166	Cell Signalling		2.6	Y
T-cell death associated gene	U44088	Cell Signalling		2.3	Y
Transforming Growth Factor Beta-3	M32745	Growth Inhibition	TGF-ß	1.9	Y
Clusterin	L08325	Apoptosis	TGF-ß	2.0	Y
Eta-1 (Osteopontin)	X16151	Cell Signalling	TGF-ß/Immune	8.8	Y
Id-2	M69293	Cell Cycle	TGF-ß	2.3	Y
Carbonic Anhydrase II	K00811	Hydratase		3.0	Y
Cyclin D1	A1849928	Cell Cycle		1.8	Y
Cellular Retinol Binding Protein 1	X60367	Retinol-Binding		3.5	Y
Connexin 26	M81445	Gap Junction		3.1	Y
Folate-Binding Protein 1	ET63126	Receptor		2.6	Y
Adenosine Deaminase	M10319	Nucleotide Metabolism		3.0	Y
Chitinase 3-like 1 (BRP-39)	X93035	Glycoprotein		3.2	Y

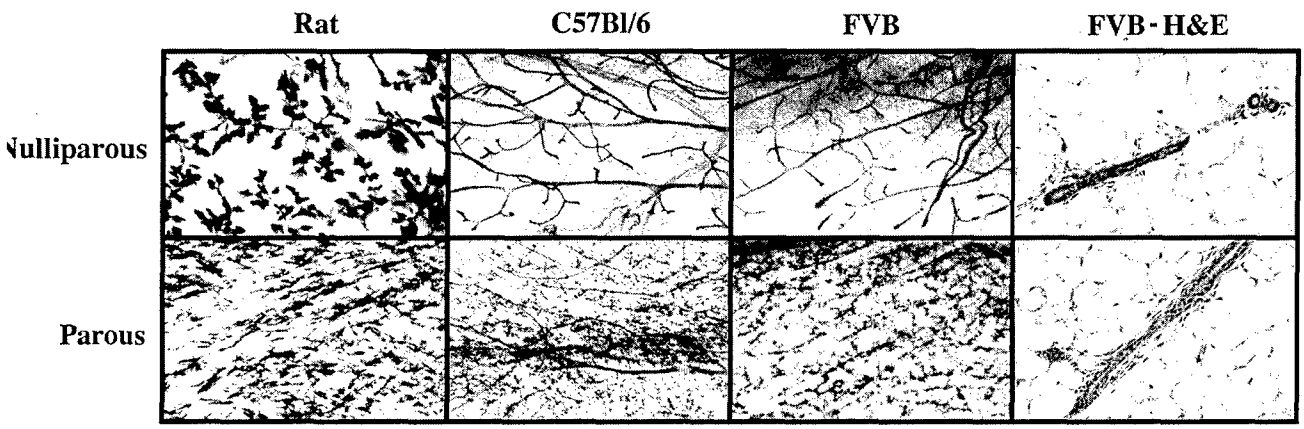
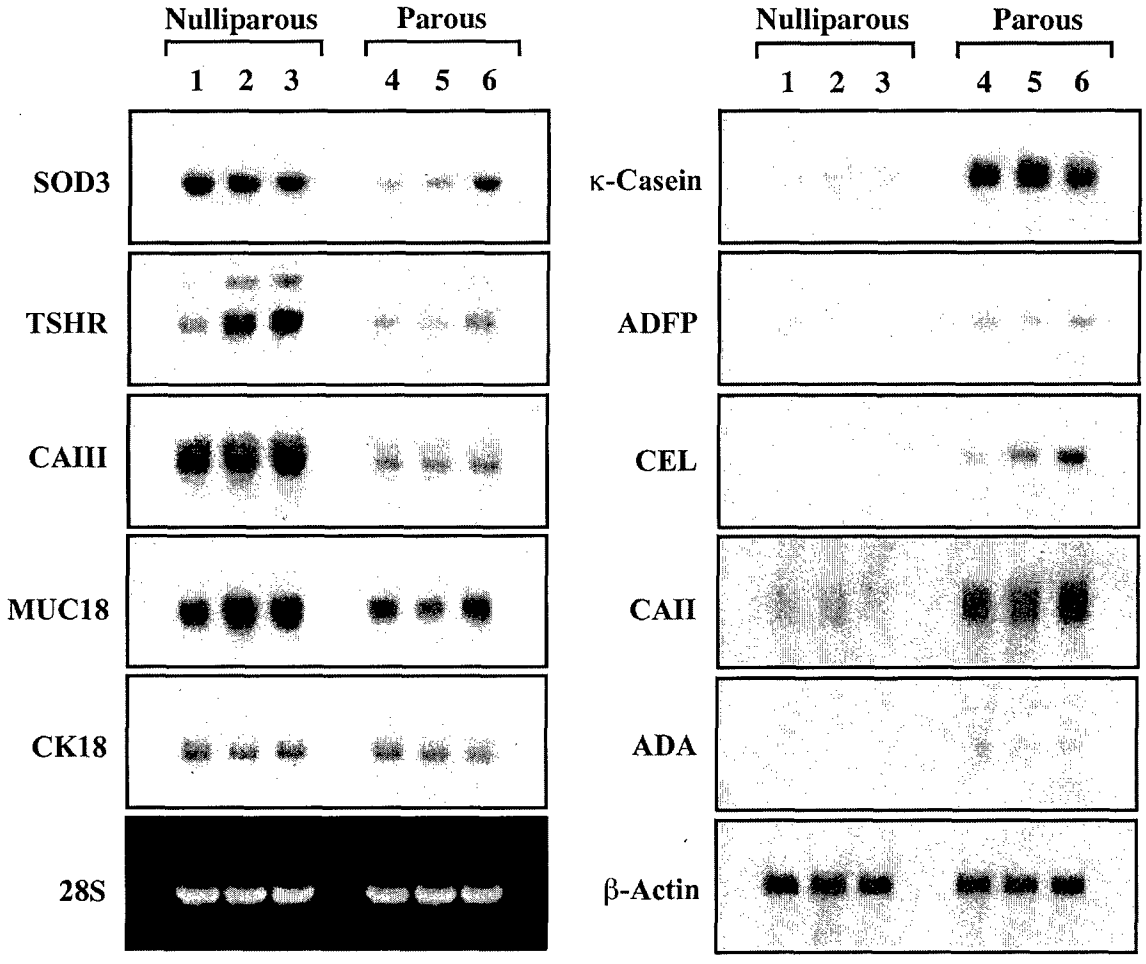


Fig. 1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TSHR
CAIII
MUC18
CK18
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Parous
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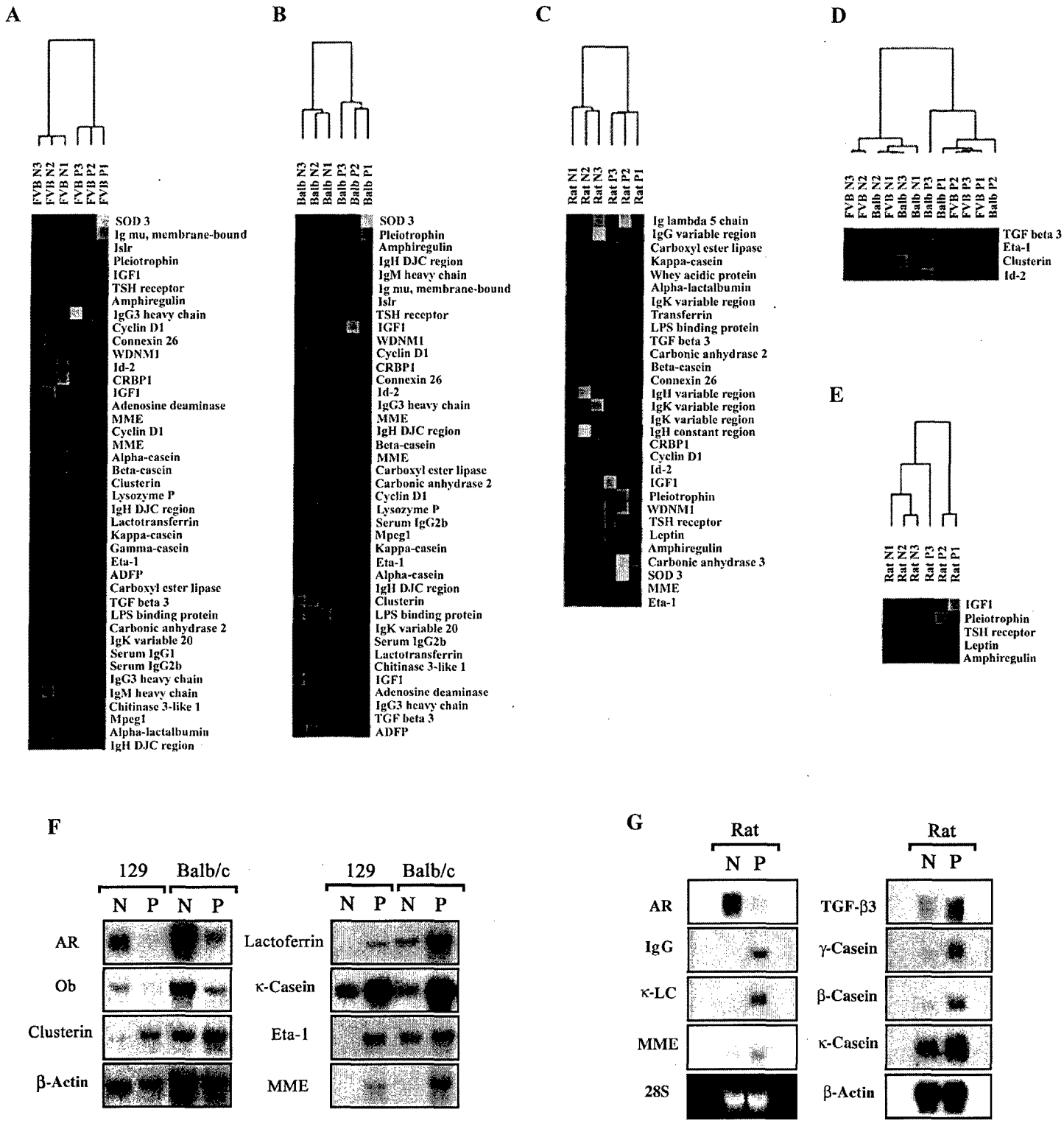
4 5 6
κ-Casein
ADFP
CEL
CAII
ADA
β-Actin

Fig. 2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FVB N2
FVB N1
FVB P3
FVB P2
FVB P1
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Islr
Pleiotrophin
IGF1
TSH receptor
Amphiregulin
IgG3 heavy chain
Cyclin D1
Connexin 26
WDNM1
Id-2
CRBP1
IGF1
Adenosine deaminase
MME
Cyclin D1
MME
Alpha-casein
Beta-casein
Clusterin
Lysozyme P
IgH DJC region
Lactotransferrin
Kappa-casein
Gamma-casein
Eta-1
ADFP
Carboxyl ester lipase
TGF beta 3
LPS binding protein
Carbonic anhydrase 2
IgK variable 20
Serum IgG1
Serum IgG2b
IgG3 heavy chain
IgM heavy chain
Chitinase 3-like 1
Mpeg1
Alpha-lactalbumin
IgH DJC region
B
Balb N3
Balb N2
Balb N1
Balb P3
Balb P2
Balb P1
SOD 3
Pleiotrophin
Amphiregulin
IgH DJC region
IgM heavy chain
Ig mu, membrane-bound
Islr
TSH receptor
IGF1
WDNM1
Cyclin D1
CRBP1
Connexin 26
Id-2
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IgH DJC region
Beta-casein
MME
Carboxyl ester lipase
Carbonic anhydrase 2
Cyclin D1
Lysozyme P
Serum IgG2b
Mpeg1
Kappa-casein
Eta-1
Alpha-casein
IgH DJC region
Clusterin
LPS binding protein
IgK variable 20
Serum IgG2b
Lactotransferrin
Chitinase 3-like 1
IGF1
Adenosine deaminase
IgG3 heavy chain
TGF beta 3
ADFP
C
Rat N1
Rat N2
Rat N3
Rat P3
Rat P2
Rat P1
Ig lambda 5 chain
IgG variable region
Carboxyl ester lipase
Kappa-casein
Whey acidic protein
Alpha-lactalbumin
IgK variable region
Transferrin
LPS binding protein
TGF beta 3
Carbonic anhydrase 2
Beta-casein
Connexin 26
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IgK variable region
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IgH constant region
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IGF1
Pleiotrophin
WDNM1
TSH receptor
Leptin
Amphiregulin
Carbonic anhydrase 3
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Balb N1
Balb P3
Balb P1
FVB P2
FVB P3
FVB P1
Balb P2
TGF beta 3
Eta-1
Clusterin
Id-2
E
Rat N1
Rat N2
Rat N3
Rat P3
Rat P2
Rat P1
IGF1
Pleiotrophin
TSH receptor
Leptin
Amphiregulin
F
129
Balb/c
N P N P
AR
Ob
Clusterin
β-Actin
129
Balb/c
N P N P
Lactoferrin
κ-Casein
Eta-1
MME
G
Rat
N P
AR
IgG
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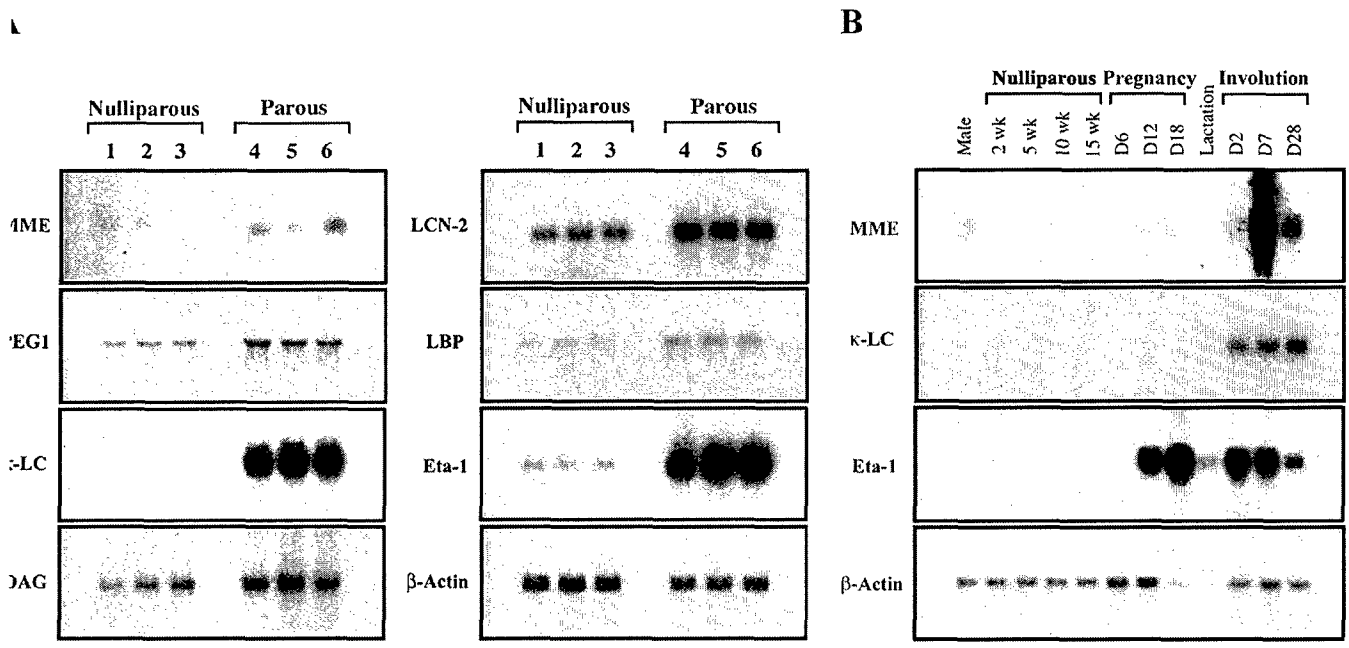
28S
Rat
N P
TGF-β3
γ-Casein
β-Casein
κ-Casein
β-Actin

Fig. 3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4 5 6
LCN-2
LBP
Eta-1
β-Actin
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Nulliparous Pregnancy
Involution
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
MME
κ-LC
Eta-1
β-Actin

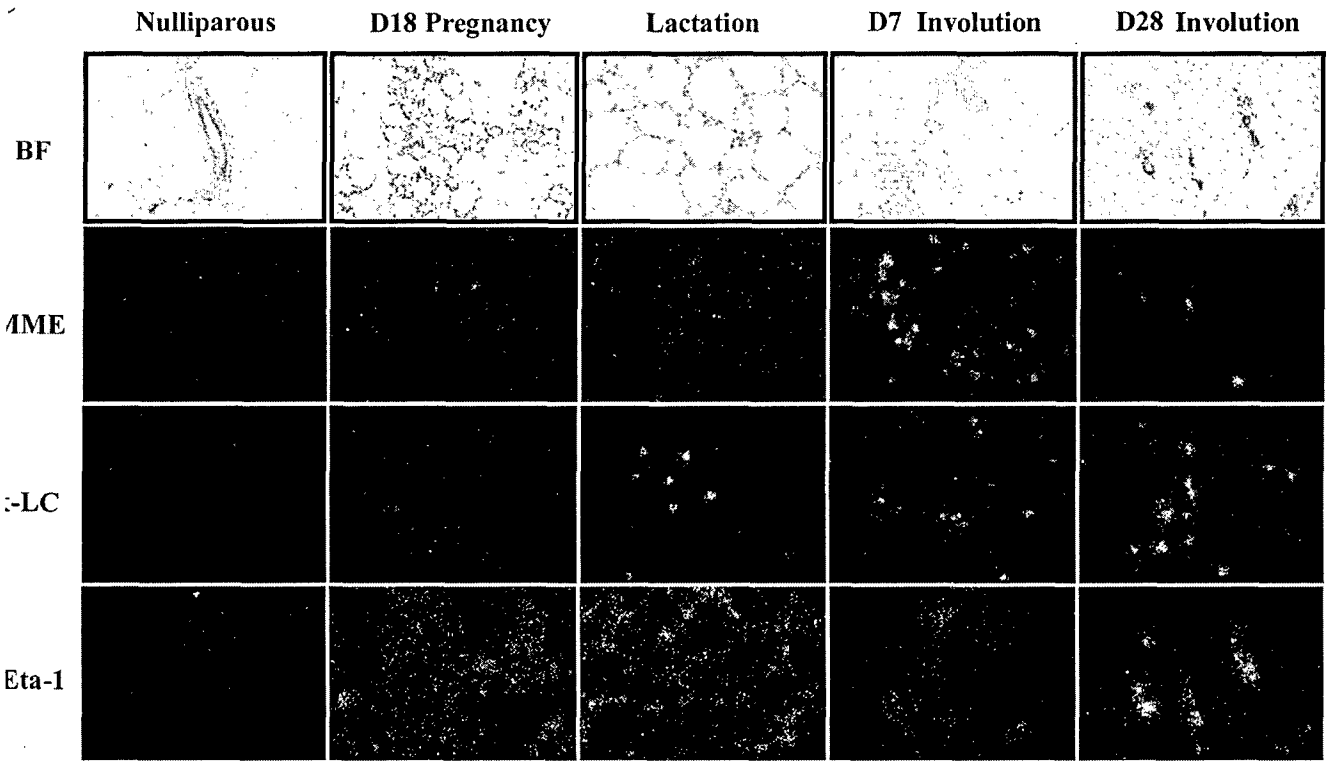

Nulliparous
D18 Pregnancy
Lactation
D7 Involution
D28 Involution
BF
Eta-1

Fig. 4

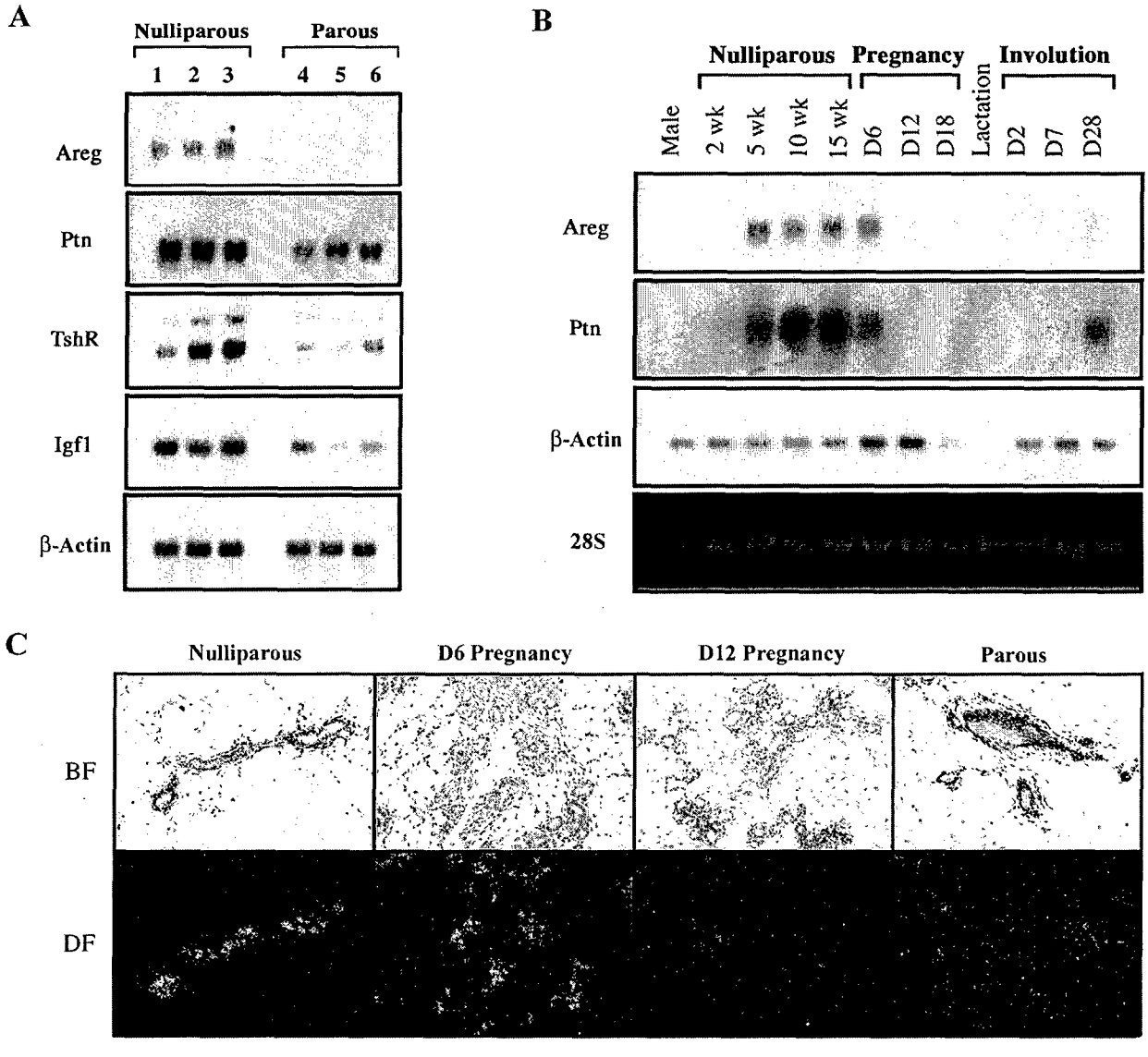
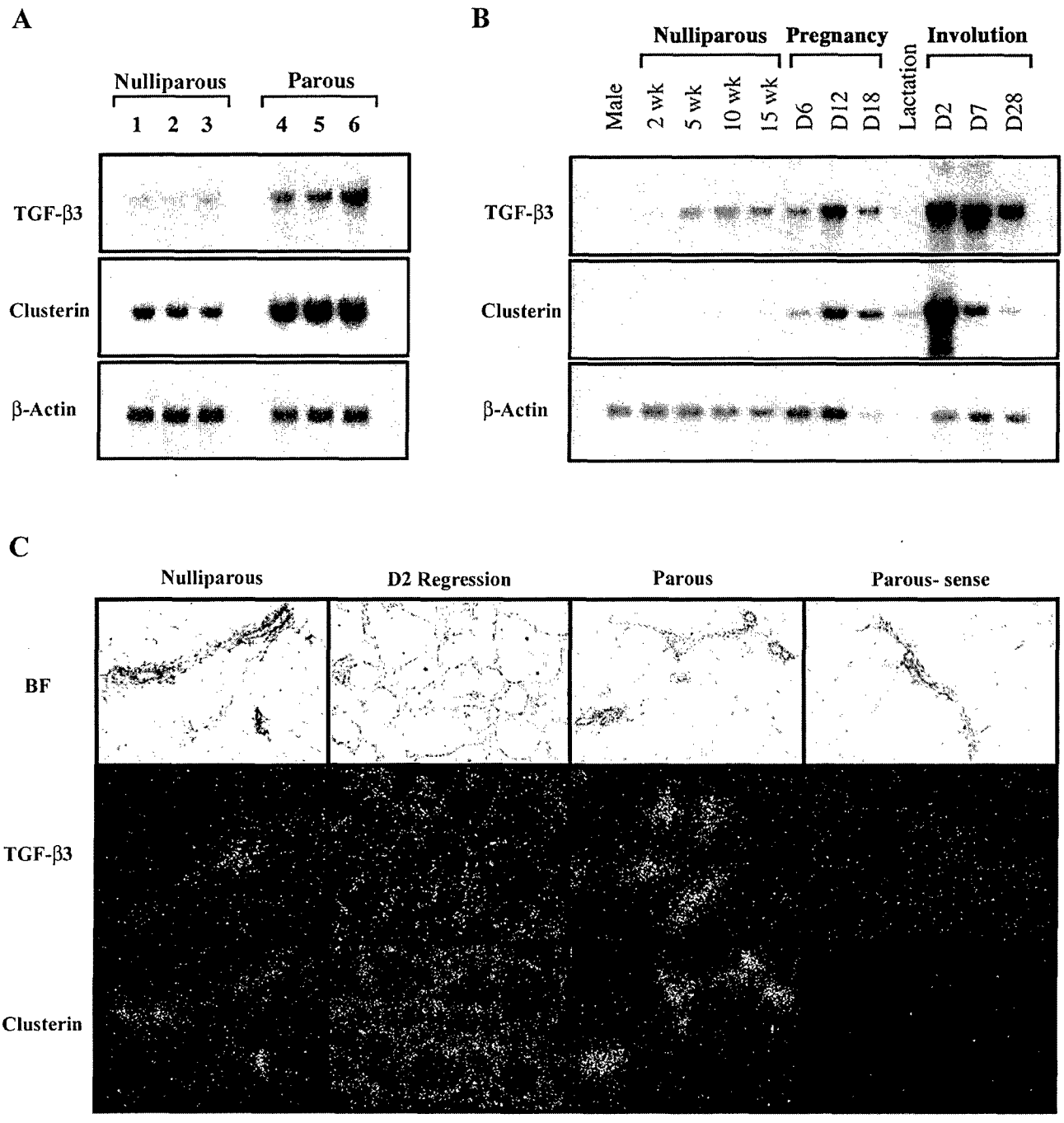

A
Nulliparous
Parous
1 2 3
4 5 6
Areg
Ptn
TshR
Igf1
β-Actin
B
Nulliparous
Pregnancy
Involution
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
Areg
Ptn
β-Actin
28S
C
Nulliparous
D6 Pregnancy
D12 Pregnancy
Parous
BF
DF

Fig. 5


A
Nulliparous
Parous
1 2 3
4 5 6
TGF-β3
Clusterin
β-Actin
B
Nulliparous
Pregnancy
Involution
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
TGF-β3
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β-Actin
C
Nulliparous
D2 Regression
Parous
Parous- sense
BF
TGF-β3
Clusterin

Fig. 6

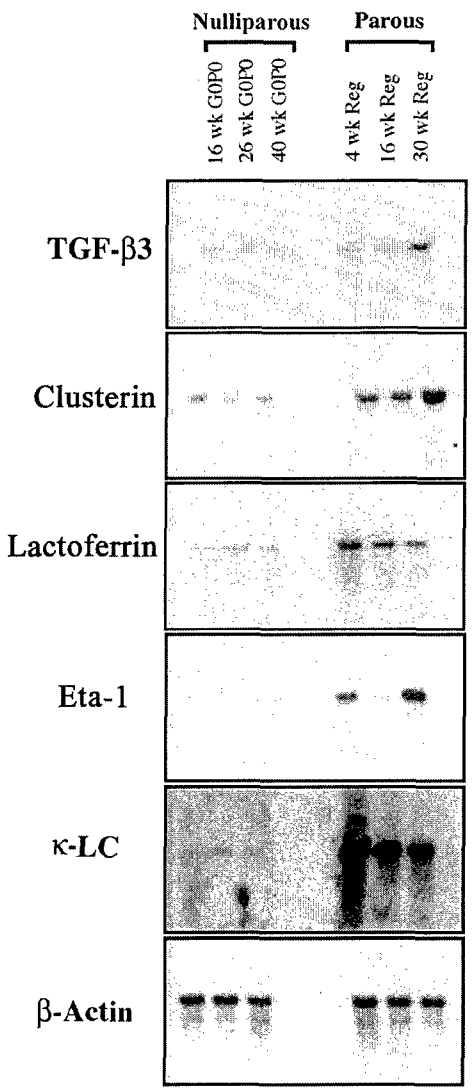
Nulliparous
Parous
16 wk G0P0
26 wk G0P0
40 wk G0P0
4 wk Reg
16 wk Reg
30 wk Reg
TGF-β3
Clusterin
Lactoferrin
Eta-1
κ-LC
β-Actin

Fig. 7

Propreitary Data

Parity-Induced Differentiation is Dependent on Reproductive History

Celina M. D'Cruz, Seung I. Ha, James Y. Wang, James D. Cox, Sandra T. Marquis and Lewis A. Chodosh

ABSTRACT

An early first full-term pregnancy in humans is one of the most effective natural protections against breast cancer. Similarly, rodents that undergo a full-term pregnancy are less susceptible to carcinogen-induced mammary tumorigenesis than are age-matched nulliparous animals. One of the prevailing hypotheses regarding the mechanism underlying this protective effect proposes that parity results in a more differentiated mammary gland that is thereby less susceptible to breast cancer. In this report, we provide the first molecular evidence supporting the hypothesis that parity results in a permanent increase in the differentiated state of the mammary gland. Specifically, we demonstrate that an early first full-term pregnancy in rodents results in the permanent upregulation of mammary epithelial differentiation markers, including β-, γ-, κ-, and ε-*casein, lactoferrin, whey acidic protein, WDNM1* and *α-lactalbumin*. In addition, the temporal regulation of these differentiation markers during pregnancy indicates that these genes define early *(γ-casein, β-casein*, and *WDNM1*), intermediate *(α-lactalbumin, κ-casein, lactoferrin*, and *WAP*), and late *(ε-casein*) stages of mammary epithelial differentiation. Since, unlike early first full-term pregnancy, late first full-term pregnancy is not protective against breast cancer, we investigated whether parity-induced changes in expression of differentiation markers was sensitive to the timing of first full-term pregnancy in mice. Interestingly, while intermediate and late differentiation markers were upregulated as a consequence of either early or late first full-term pregnancy, early markers of mammary epithelial differentiation were only elevated as a consequence of early first full-term pregnancy. As such, the early differentiation markers, *γ-casein, β-casein*, and *WDNM1* represent the first genes whose expression reflects events specific to the timing of a first full-term pregnancy.

MATERIALS AND METHODS

Animals and Tissues

FVB and BALB/c mice were housed under barrier conditions with a 12 hr light/dark cycle and access to food and water *ad libitum.* Parous rodents were generated by mating 4 week, 19 week, or 25 week animals, allowing 21 days of lactation, and 4 weeks of post-lactational involution. For longer regression times, parous animals were mated at 4 weeks of age, allowed to lactate for 21 days, followed by 4 weeks, 16 weeks, or 30 weeks of post-lactational involution. At the time of harvest, mammary glands #3-5 were harvested from parous and age-matched nulliparous littermates and snap frozen. Lymph nodes within mammary gland # 4 were visualized and removed.

RNA Isolation and Northern Analysis

Snap-frozen tissue was homogenized in guanidine thiocyanate supplemented with 7 ul/ml of β-mercaptoethanol, and RNA was isolated by centrifugation through cesium chloride as previously described. RNA was quantitated and equal amounts from each sample were used to generate independent pools. Total RNA was separated on a 1% LE agarose gel, passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described (Marquis et al., 1995).

In Situ Hybridization

In situ hybridization was performed as described (Marquis et al., 1995). Antisense and sense probes were synthesized with the Promega in vitro transcription system using 35S-UTP and 35S-CTP from the T7 and SP6 RNA polymerase promoters

of a PCR template containing the sequences used for Northern Hybridization analysis.

INTRODUCTION

Epidemiologic studies have consistently demonstrated that reproductive history influences breast cancer risk. This relationship is illustrated by the observation that women who undergo an early first full-term pregnancy have a significantly reduced life-time risk of breast cancer (Bain et al., 1981; Carter et al., 1989; Ewertz et al., 1990; Helmrich et al., 1983; Layde et al., 1989; Leon, 1989; Lubin et al., 1982; Lund, 1991; MacMahon et al., 1970; MacMahon et al., 1982; Negri et al., 1988; Paffenbarger et al., 1980; Rosner and Colditz, 1996; Rosner et al., 1994; Salber et al., 1969; Tulinius et al., 1978; Wang et al., 1992; Yuan et al., 1988). However, the protection conferred by first childbirth diminishes as a woman's age at first childbirth increases. In fact, a first full-term pregnancy after the age of 30 increases a woman's risk of breast cancer as compared to women who never have children. Thus, the *timing* of a first full term pregnancy has a greater impact on breast cancer risk than its occurrence *per se*. At present, the biological basis of parity-induced protection is unknown. Nevertheless, the finding that breast cancer risk is altered in a predictable fashion by specific reproductive events raises the possibility that protective events such as an early full-term pregnancy could be mimicked pharmacologically. In order to achieve this goal, however, the underlying molecular events that influence breast cancer risk must be defined. In addition, the identification of molecular markers that accurately reflect the biological changes in the breast associated with an alteration in breast cancer susceptibility would provide a molecular basis by which to assess the efficacy of such regimens. Unfortunately, at present there is a paucity of information regarding genes whose expression reflects specific states of the

breast that correlate with breast cancer susceptibility. As such, a comprehensive molecular description of the protected mammary gland will ultimately aid in the design and implementation of chemoprevention protocols designed to mimic the naturally occurring protective effects of early parity.

Although the mechanisms underlying parity-induced protection from breast cancer are unknown, several hypotheses have been proposed. In principle, this protective effect could result from the pregnancy-driven terminal differentiation of a subpopulation of target cells or from the preferential loss of a subpopulation of target cells during post-lactational involution. In addition, permanent systemic endocrine changes could affect the breast in such a way as to reduce the risk of carcinogenesis. Current evidence suggests that aspects of both models may be valid; expression levels for the estrogen receptor and epidermal growth factor receptor are reported to decrease in the parous epithelium, whereas changes in circulating hormone levels have also been reported to occur as a consequence of parity (Musey et al., 1987; Thordarson et al., 1995). Thus, it appears that both systemic and local events in the breast may cooperate to reduce the susceptibility to carcinogenesis in the parous breast.

The majority of data on parity-induced protection against breast cancer have been garnered from studies of rodent models that, like humans, display a decreased susceptibility to mammary tumorigenesis following an early full-term pregnancy (Dao et al., 1960; Huggins et al., 1959; Medina and Smith, 1999; Medina and Smith, 1999; Moon,

1981; Moon, 1969; Russo and Russo, 1978; Russo et al., 1977; Russo et al., 1979)].

Importantly, developmental processes and morphological features of the rodent mammary gland are also very similar to those of the human breast. As in humans, ductal morphogenesis in rodents is initiated by club-like structures, referred to as terminal end-buds (TEB), that contain the progenitor cells required for ductal elongation and morphogenesis (Daniel and Silberstein, 1987; Russo et al., 1990). As the mammary gland matures, TEB regress into terminal duct (TD) structures or develop into alveolar buds (AB) either with age or at the onset of pregnancy (Daniel and Silberstein, 1987; Russo et al., 1990). The hypothesis that parity induces the differentiation of mammary epithelium has been studied at the morphological level in Sprague-Dawley rats (Russo and Russo, 1978; Russo et al., 1982). Russo *et al.* have demonstrated that the parous mammary gland is more differentiated based on morphological features, than the nulliparous mammary gland (Russo and Russo, 1978; Russo et al., 1982). By quantifying the relative abundance of terminal end buds (TEB), terminal ducts (TD), and alveolar buds (AB), Russo *et al.* determined that the parous epithelial tree consists of a greater proportion of the more differentiated AB structures, whereas the nulliparous epithelium is composed primarily of the less differentiated TD structures (Russo and Russo, 1978; Russo et al., 1982). Moreover, analysis of proliferation rates within TEB, TD and AB indicate that TEB have the highest rate of proliferation whereas the lowest rates are seen in AB. These results suggest that epithelial cells within morphologically differentiated structures are associated with a reduced proliferation rate (Russo and Russo, 1978; Russo et al., 1982). As such, these observations suggest that parity induces morphological differentiation of

the mammary gland, and that this stage is associated with reduced epithelial proliferation rates and correlates with a reduced susceptibility to carcinogenesis.

Recently, we have identified a panel of genes that are differentially expressed between the nulliparous and parous murine mammary gland (D'Cruz, manuscript in preparation). In particular, several markers of mammary epithelial differentiation were permanently upregulated as a consequence of parity. These genes include several milk protein genes, *α-casein, β-casein, γ-casein, κ-casein, whey acidic protein (WAP), α-lactalbumin* and *WDNM1*. While lactation is a parity-related event that ultimately leads to terminal differentiation of mammary epithelial cells concomitant with an upregulation of milk protein gene expression, is it unclear to what extent such differentiation events persist in a fully involuted mammary gland. Thus, the finding that differentiation markers are expressed at higher levels in the parous mammary gland as compared to the nulliparous mammary gland suggests that parity has resulted in a more differentiated mammary gland.

Milk protein gene expression has previously been reported to parallel the differentiation status of the breast (Robinson et al., 1995). Prior to pregnancy, the nulliparous mammary gland is morphologically undifferentiated, and is accompanied by low levels of expression of milk protein genes (Robinson et al., 1995). During pregnancy, mammary alveoli are formed and develop into lobules that ultimately become fully differentiated secretory structures during lactation (Pitelka et al., 1973). Increased

synthesis of a variety of milk protein genes accompanies each of these morphological stages. Moreover, in animal models in which the misexpression of a transgene results in the inability of these animals to lactate, this block in differentiation is accompanied by a gross reduction in milk protein expression levels (Robinson et al., 1995).

Although milk protein genes are globally regulated by lactogenic hormones, each gene responds in a unique manner to the hormonal environment of pregnancy (Burdon et al., 1991; Pittius et al., 1988; Shamay et al., 1992). For example, the *β-casein* and *WAP* promoters both contain response elements for steroid hormones and the transcription factor STAT 5 (Li and Rosen, 1994; Wakao et al., 1994). Despite this similarity in promoter elements, however, *β-casein* is upregulated by prolactin and, to a lesser extent, by glucocorticoids, while *WAP* responds more strongly to glucocorticoids than to prolactin (Burdon et al., 1991; Pittius et al., 1988; Shamay et al., 1992). Based on hormonal regulation during pregancy, high prolactin levels and *β-casein* expression occur during early pregnancy while high glucocorticoid levels and *WAP* expression are associated with late pregnancy (Robinson et al., 1995). As such, milk protein genes represent a broad category of markers for differentiation that are regulated by independent mechanisms, and thus mark different stages of differentiation in the mammary gland.

In this report, we have investigated the relative distribution of cells expressing mammary epithelial differentiation markers in the nulliparous and parous murine mammary gland. In addition, we have analyzed the temporal regulation of these genes

during pregnancy. Our findings demonstrate that these genes are responsive to different hormonal cues and can readily be distinguished as early, intermediate, and late markers of mammary epithelial differentiation. We have used this panel of differentiation markers to address the differentiation status of the mammary gland following reproductive variables relevant to parity. In doing so, we have demonstrated that the parity-dependent expression of early markers of differentiation is sensitive to the timing of first full-term pregnancy.

RESULTS

Differentiation Markers are Permanently Upregulated as a Result of Early Parity

We previously used oligonucleotide microarray analysis to identify genes that are differentially expressed between the mammary glands of nulliparous and parous mice. This approach identified eight milk protein genes whose expression increases as a consequence of parity (D'Cruz, manuscript in preparation). Since milk protein genes are markers of mammary epithelial cell differentiation, this finding suggests that parity results in a more differentiated mammary gland. Although the parous mammary gland has been described as being more differentiated morphologically than age-matched nulliparous glands, to date there has been no molecular evidence to support this proposal. In an effort to examine the extent of parity-induced differentiation at both the molecular and cellular level, we have investigated the impact of reproductive history on the expression of several milk protein genes in the murine mammary gland.

To confirm the preferential expression of milk protein genes in the parous mammary gland as revealed by microarray analysis, we have cloned cDNA fragments for β-, γ-, κ-, and ε-*casein, lactoferrin, whey acidic protein, WDNM1* and *α-lactalbumin.* Labeled probes for *β-casein, γ-casein, κ-casein* and *lactoferrin* were hybridized to Northern membranes containing total RNA isolated from nulliparous and parous mammary glands harvested from independent animals. In this experiment, parous animals underwent a single round of pregnancy, lactation, and 28 days of post-lactational involution. Animals were mated at 4 weeks of age, a time point that corresponds to an early stage of puberty in FVB mice, resulting in a mating that is analogous to early parity in humans. Age-matched nulliparous littermates served as controls. By Northern analysis, steady state mRNA levels for *β-, γ-,* and *κ-casein,* and *lactoferrin* were easily detectable in both nulliparous and parous mice (Fig. 1A and data not shown). Transient milk protein gene expression has previously been reported in nulliparous mice during the estrus cycle and may underlie the animal-to-animal variability in expression observed in this study (Robinson et al., 1995). To mimimize the impact of this variablility on our analysis, we pooled equivalent amounts of mRNA from 15-20 mice at each reproductive stage examined. We detected a 4-fold or greater increase in expression of each differentiation marker in parous as compared to nulliparous glands.

In contrast to the expression *of β-, γ-,* and *κ-casein*, and *lactoferrin*, mRNA levels for *WDNM1, α-lactalbumin, WAP* and *ε–casein* were low to undetectable by Northern analysis of total RNA isolated from nulliparous glands. As such, parity-dependent changes in expression were examined for these milk protein genes by Northern analysis of

purified poly(A)+ mRNA from pooled samples of 15-20 nulliparous and parous mice (Fig. 1B). These data confirm the differential expression of markers for mammary epithelial cell differentiation in the parous mammary gland following an early first full-term pregnancy.

The Population of Differentiated Epithelial Cell Types is Expanded by Parity

The upregulation of epithelial differentiation markers in the parous mammary gland could occur as a result of a global increase in gene expression on a per-cell basis, an increase in the percentage of expressing cells, or both. To determine which of these potential mechanisms determines the basis for the parity-dependent upregulation of milk protein gene expression, we performed *in situ* hybridization to define the spatial pattern of expression for milk protein genes in the nulliparous and parous mammary gland. This analysis revealed that the nulliparous mammary gland contains a small number of cells capable of expressing *β-casein* at relatively high levels (Fig. 2). In contrast, the parous mammary gland exhibited a marked increase in the proportion of epithelial cells expressing *β-casein*, and each expressing cell was found to express *β-casein* at levels similar to those detected in expressing cells in the virgin gland (Fig. 2 and data not shown). The expression of β-casein was principally confined to alveolar buds and was largely variable among adjacent cells within these structures. *κ-casein, lactoferrin* and *WDNM1* expression was readily detectable in both ducts and alveolar ductules of nulliparous and parous mammary glands (Fig. 2 and data not shown). Similar to *β-casein,* the number of epithelial cells expressing these genes were each expanded in the parous epithelium as

compared to nulliparous epithelium (Fig. 2). In addition, the intensity of expression for each marker exhibited marked variation for adjacent cells, indicating that there is heterogeneity among epithelial cells.

Consistent with the low levels of *ε-casein* and *WAP* mRNA expression detected by Northern analysis, expressing cells were not detected by *in situ* hybridization performed on sections of nulliparous mammary gland tissue. In comparison, 1-2 cells expressing *ε-casein* or *WAP* were detected per field in the parous mammary gland by *in situ* analysis (data not shown). In aggregate, these data suggest that the primary mechanism underlying the increase in expression of epithelial differentiation markers is an increase in the percentage of cells expressing these markers in the parous mammary gland. Moreover, the heterogeneity of milk protein gene expression observed among adjacent cell types suggests either that epithelial cells are responding to subtle differences in the local environment within the mammary gland, or that distinct epithelial cell sub-types or states exist within the mamamary gland, or both.

Early, Intermediate and Late Markers of Mammary Epithelial Differentiation

We reasoned that regulatory distinctions are likely to exist among different members of the milk protein family and that these markers might provide markers for different stages of cellular differentiation and, therefore, provide insight into the differentiation state of the mammary epithelium. Although all milk protein genes are upregulated by lactogenic hormones during pregnancy and lactation, important differences in temporal regulation

have been reported (Burdon et al., 1991; Pittius et al., 1988; Shamay et al., 1992). Specifically, the expression of *β-casein* and *WDNM1* in this study was first detected by *in situ* hybridization in the alveolar epithelial cells at day 9 of pregnancy, whereas *α-lactalbumin* and *WAP* were not detected until day 14 and day18 of pregnancy, respectively (Robinson et al., 1995). Consequently, *β-casein* and *WDNM1* were considered to represent markers of early differentiation events, while *WAP* and *α-lactalbumin* were considered to represent late events in mammary epithelial differentiation (Robinson et al., 1995).

In order to investigate the temporal regulation of *β-casein* in our system, we monitored *β-casein* mRNA levels over distinct phases of mammary gland development, including nulliparous development, three trimesters of pregnancy, lactation, and early, mid and late involution. A substantial increase in *β-casein* mRNA expression was first detected at day 6 of pregnancy (Fig. 3A). Subsequently, steady state mRNA levels_for_-*casein* were found to increase throughout pregnancy with maximal expression occurring during lactation (Fig. 3A). Levels of expression decreased throughout involution yet remained elevated compared to age-matched nulliparous glands at day 28 of involution (Fig. 3A). An eight-fold increase in expression of *β-casein* was demonstrated in the parous animal (D28 involution) as compared to that of an age-matched nulliparous animal (15 wk G0P0) (Fig. 3B and 1A). This finding is consistent with a model in which cells marked by *β-casein* expression represent residual cells from the pregnancy-induced

expansion and differentiation of mammary epithelium, and that have been maintained following involution.

Of note, we have detected the onset of *β-casein* expression at an earlier point in pregnancy than previously reported (Robinson et al., 1995). This finding may reflect either the increased sensitivity of our methods of detection, or that earlier time points in pregnancy were not investigated in other studies (Robinson et al., 1995). Nonetheless, we reasoned that a temporal analysis of expression of other milk protein genes could help define a system by which to categorize distinct stages of mammary epithelial differentiation.

Probes for each of eight milk protein genes were hybridized to Northern membranes containing total RNA isolated from 10 week nulliparous controls as well as animals at days 6, 10, 14 and 18 of pregnancy (data not shown). Raw expression values were normalized to β-actin and plotted for each milk protein gene (Fig. 4A). As expected, expression levels of each milk protein gene increased over nulliparous levels at some point during the course of pregnancy (Fig. 4A). The expression profiles for each gene were subsequently normalized to expression levels in nulliparous animals (Fig. 4B). Differences in rates of increase in gene expression levels for each marker suggested that the regulation of these genes differ dramatically. While *β-casein* and *γ-casein* expression increases sharply during the early stages of pregnancy, expression of *κ-casein* increases at a much slower rate (Fig. 4B). In addition, the highest levels of expression achieved during

pregnancy varied considerably between milk protein genes. WAP and *γ-casein* expression increased approximately 1,000-fold by the end of pregnancy, whereas *ε-casein* demonstrated only a 10-fold increase in expression (Fig. 4B). Each milk protein gene attained its maximal level of expression during lactation, a stage at which the epithelium is considered to be fully differentiated (Fig. 3A and 3B, data not shown).

Previous categorizations of markers as early, mid, or late have relied on the first stage at which expression was detected. However, this type of analysis is inevitably influenced by the sensitivity of the particular detection method employed. As such, methods of detection with lower sensitivity would tend to classify two markers with identical temporal patterns of expression differently if the absolute expression levels of these markers differed. That is, a gene that is expressed at a lower level would tend to be classified as a later marker than a gene expressed at a high level, since a more poorly expressed gene will not be detected until a later stage in pregnancy. In order to circumvent this analytical problem, we postulated that by using very sensitive methods of detection and by plotting the rate of increase in expression from one stage of pregnancy to the next, we would be able to identify the developmental stage at which the greatest increase in steady state mRNA levels occurred.

Using the analysis above, *γ-casein, β-casein*, and WDNM1 all were found to undergo the largest increase in expression from nulliparity to D6 of pregnancy (Fig. 4C and 5). Although *β-casein* levels continued to increase from D6 to D10, this increase was

3-fold as compared to 14-fold in the earlier interval (Fig. 4C). α-lactalbumin demonstrated similar rates of increase in gene expression from day 6 to day 10 and from day 10 to day 14 (Fig. 4C). Lactoferrin and κ-casein underwent modest increases in gene expression from D10 to D14, while WAP demonstrated a dramatic change in the rate of increase over this same period (Fig. 4C and 5). Furthermore, an increase in _-casein expression was first detected at D14 of pregnancy but the largest increase in expression occurred between day 14 and day 18 (Fig 4C and 5). In contrast, expression of GAPDH, and cytokeratin 18 (CK18), a marker of epithelial cells, did not appear to increase during pregnancy (Fig. 4C). Based on the rate of increase for each marker throughout pregnancy, *γ-casein*, *β–casein* and *WDNM1* were classified as early markers of differentiation in the mammary gland. Similarly, α-lactalbumin, lactoferrin, κ-casein and WAP were classified as intermediate markers of differentiation. Finally, since the most robust change in regulation of ε-casein was not initiated until the last trimester of pregnancy, this gene was classified as a late marker of mammary epithelial differentiation.

Spatial Expression of Differentiation Markers During Pregnancy

In order to confirm the biological validity of this method of analysis, we analyzed the temporal regulation of*β-casein*, *WAP* and *ε-casein* mRNA during pregnancy by *in situ* analysis. We reasoned that if a classification of markers based on the rate of change in expression was valid, the same classification would be made using an independent analytical approach. By in situ hybridization, *β-casein* expression was low to barely detectable in mammary gland sections from 10 week nulliparous animals, yet was markedly induced in both ducts and alveolar cells by day 7 of pregnancy (Fig. 5). This expression was initially punctate throughout the epithelium and became more uniform in its expression in adjacent cells by day 14 and day 18 of pregnancy. In comparison, *WAP* expression was not detectable in sections from nulliparous animal or at day 7 of pregnancy, but was easily detected at day 14 of pregnancy (Fig. 5). Finally, the induction of *ε-casein* expression during late pregnancy is demonstrated by the punctate increase in expression that first appeared at day 14 of pregnancy and became more uniform and greater in magnitude by day 18 of pregnancy (Fig. 5). In aggregate, these data suggest that *β-casein, WAP* and *ε-casein* mark distinct temporal stages of proliferation and differentiation that occur as mammary epithelial cells differentiate.

Early Markers of Differentiation are Sensitive to Age at Mating

Given the fact that reproductive history and susceptibility to mammary carcinogenesis are intrinsically related, we wished to address the question of whether parity-induced changes in mammary differentiation was correlated with the changes in

breast cancer susceptibility that are associated with different developmental stages. To address this question, we generated cohorts of rats and of mice of different strains that had different reproductive histories with respect to parity, timing of a first full-term pregnancy, and duration of regression. Upregulation of milk protein gene expression as a consequence of early first full-term pregnancy was observed in total RNA isolated from the mammary glands of nulliparous and parous Sprague-Dawley rats and Balb/c mice (Fig. 6A, data not shown). These results established that the preferential expression of differentiation markers in the mammary gland of parous animals was not species-specific or strain-specific.

We next reasoned that if parity permanently induced differentiation following an early first full-term pregnancy, 3 weeks of lactation, and 4 weeks of involution, these changes in differentiation would persist at longer points of involution. To test this hypothesis, we generated cohorts of animals that underwent an early mating, lactation and 16 weeks of post-lactational involution. The expression of each of the milk protein genes examined in this study demonstrated a parous-specific pattern of expression independent of length of regression (Fig. 6B, data not shown). This result suggests that the changes in the differentiated state of the mammary gland as a result of parity is maintained over longer periods of time in the parous mammary gland.

Epidemiologic studies have shown that the timing of a first-full term pregnancy is critical for establishing a woman's lifetime risk of breast cancer. For this reason, we

wished to examine whether the timing of a first full-term pregnancy was an important factor in determining the extent of parity-induced differentiation. As such, the expression of differentiation markers in the mammary glands of animals mated at 4 weeks of age, 19 weeks of age, and 25 weeks of age were examined. In each case, animals underwent a single pregnancy followed by 21 days of lactation and 28 days of post-lactational involution, and were compared to age-matched nulliparous controls. By this analysis, parity-dependent upregulation of expression of *lactoferrin, κ-casein, α-lactalbumin*, and *WAP* were found to be independent of age at mating in that they were upregulated following either an early first full-term pregnancy (Fig. 1A and B) or a late first full-term pregnancy (Fig. 6C and Fig. 7, data not shown).

Surprisingly, the expression of the early differentiation markers, *β-casein, γ-casein,* and *WDNM1* in parous animals was sensitive to the age at first full-term pregnancy (Fig. 7). The level of expression of these early markers of differentiation is actually lower in late-mated parous animals compared to age-matched nulliparous controls. The inverse patterns of parity-dependent expression of early differentiation markers for early versus late first full-term pregnancy appear to be due to age-dependent increases in expression of *β-casein, γ-casein*, and *WDNM1* in nulliparous animals coupled with slight decreases in parous animals mated at a later age (data not shown). To our knowledge, these represent the first genetic markers in the mammary gland whose expression varies with changes in reproductive history that are known to alter breast cancer risk.

DISCUSSION

In this report we have provided molecular evidence to support the hypothesis that parity results in a more differentiated mammary gland. Previously, the differentiation status of the mammary gland has been assessed based on morphologic criteria and proliferation rates (Russo and Russo, 1978; Russo et al., 1982). As such, the panel of differentiation markers identified in this study represents the first molecular description of the differentiated states of the nulliparous and parous murine mammary gland. Furthermore, we have identified molecular markers for epithelial differentiation that are sensitive to reproductive endocrine events that alter breast cancer risk.

The panel of differentiation markers described in this study represent discrete stages of mammary epithelial differentiation, including early, intermediate and late events. The use of flourescently labeled oligonucleotides for *in situ* hybridization has demonstrated that during lactation several different milk protein genes are expressed within a single alveolar cell (Joseph and Collet, 1994). This suggests that terminally differentiated mammary epithelial cells are competent to express all milk protein genes. Based on this data, we hypothesize that the panel of differentiation markers used in this study will have overlapping expression within a given cell, such that cells expressing *ε-casein* would also express intermediate and early markers of epithelial differentiation. Thus, the fact that only a small percentage of parous epithelial cells expresses *ε-casein* suggests that very few terminally differentiated cells remain in the epithelium following involution.

It is intriguing to speculate on the mechanism of the relative expansion of epithelial cells expressing early and intermediate markers in parous epithelium as compared to nulliparous epithelium following an early first full-term pregnancy. Since the early markers, *γ-casein, β-casein,* and *WDNM1* are genes expressed in epithelial cells that are rapidly dividing during early pregnancy, it is likely that these cells are still capable of responding to the hormonal cues of pregnancy and are therefore still able to undergo proliferation in the event of a subsequent pregnancy. Similarly, intermediate markers, *α-lactalbumin, κ-casein, lactoferrin*, and *WAP*, are regulated at a time when the epithelium is still undergoing proliferation and differentiation. As such, our data suggest that an early first full-term pregnancy results in the expansion of epithelial cells that have initiated a program of differentiation, but are not fully differentiated. Thus, the relative percentage of cells that remain undifferentiated in a parous mammary gland is lower than that in a nulliparous mammary gland, and may potentially result in fewer target cells for mammary carcinogenesis.

Interestingly, parity-dependent expression of intermediate and late markers of differentiation is not dependent on the timing of the first full-term pregnancy while early markers of differentiation are sensitive to this reproductive variable. That is, expression of *γ-casein, β-casein*, and *WDNM1* levels are increased in parous mammary glands as compared to nulliparous mammary glands following an early first full-term pregnancy, but not a late first full-term pregnancy. This finding suggests that a late first full-term

pregnancy results in a larger abundance of cells marked by the expression of early markers in the nulliparous gland than in the parous gland. In this regard, it has been demonstrated in rat models that the mammary glands of nulliparous animals become morphologically more differentiated with time (Russo and Russo, 1978; Russo et al., 1979). Consistent with a model in which the nulliparous mammary gland differentiates with time, we have documented an increase in milk protein gene expression throughout virgin development that is consistent with the reported increase in differentiated ductal structures (data not shown). In aggregate, these results suggest that over time nulliparous mammary gland development proceeds through the early stages of epithelial differentiation. In combination with the induction of later differentiation stages that occur during pregnancy, this nulliparous development results in a greater proportion of cells expressing early differentiation markers in the aged nulliparous mammary gland than in age-matched parous glands.

In rodent systems, it has been demonstrated that the increase in differentiation of nulliparous mammary glands is accompanied by a decrease in carcinogen-induced mammary tumorigenesis (Russo et al., 1982). To date, the tumor incidence of nulliparous and parous animals following a late mating has not been reported. This may result in part, from the very low incidence of tumors that develop in older nulliparous animals as well as tumor latencies that may exceed the life expectancy of these animals. If in fact rodent models parallel humans with respect to the effects of a late first full-term pregnancy on breast cancer risk, as they do with early first full-term pregnancy, our

findings suggest that the proportion or total number of cells marked by the expression of early markers of differentiation may correlate with breast cancer risk.

We believe that we have identified a panel of differentiation markers whose expression correlates with known reproductive factors for breast cancer risk. The expression of such genes and the relative abundance of cells marked by their expression may be informative indicators of breast cancer susceptibility. Furthermore, it is possible that the markers discussed in the study will be useful in providing a molecular endpoint to test the efficacy of chemoprevention regimens targeted at mimicking the effects of an early first full-term childbirth.

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Figure Legends

Figure 1. Early Parity Results in the Upregulation of Differentiation Markers. (A). Northern analysis *of β-casein, γ-casein, κ-casein,* and *lactoferrin* expression in 15-30 independently prepared total RNA samples from nulliparous and parous mammary glands. Expression values are normalized to *β-actin*. Error bars represent standard error of the mean. (B). Northern analysis of *WDNM1, ε-casein, α-lactalbumin*, and *WAP* expression in pooled poly(A) RNA samples of 15-20 nulliparous and parous animals. Expression levels are normalized to *β-actin* to control for loading.

Figure 2. Milk Protein Genes Mark the Expansion of Cell Types in the Parous Mammary Gland. *In situ* hybridization analysis of *β-casein, κ-casein, WDNM1*, and *lactoferrin* expression in mammary glands sections from nulliparous (15 wk G0P0) and parous (15 wk GIPI, D28 regression) mice. Bright field exposures of epithelial ducts embedded in mammary fat pad. Magnification 300x. Dark field exposures of nulliparous

and parous animals have equal exposure times to facilitate comparison. Magnification 300x.

Figure 3. *β-casein* mRNA Levels Remains Elevated in the Parous Mammary Gland. (A). Northern hybridization of *β-casein* to pools of total RNA from the indicated developmental time points. β-Actin is shown to visualize the dilution effect that occurs during late pregnancy and lactation due to large scale production of milk genes. (B). Bar graph of A. Note the 8 fold increase in expression of *β-casein* in the parous mammary gland (15 wk G1P1, D28 regression) as compared to an age-matched nulliparous control (15 wk G0P0).

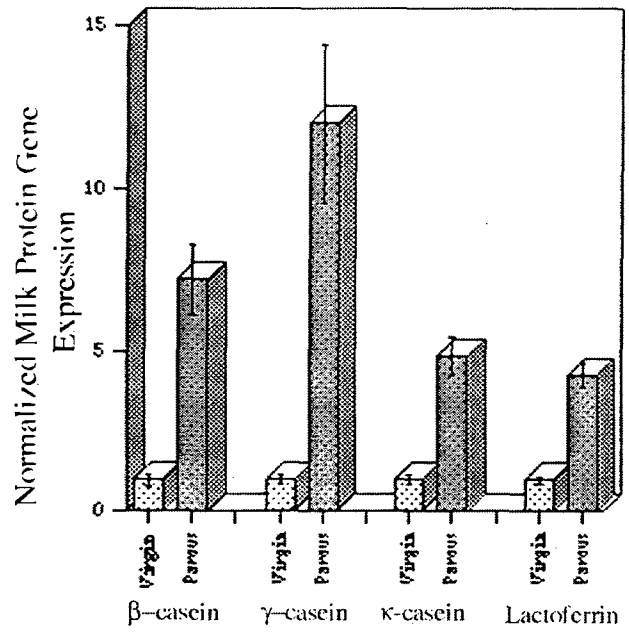
Figure 4. Milk Protein Genes are Markers for Early, Intermediate and Late MEC Differentiation Events. (A). Line graphs of raw expression levels for*β-casein*, *ε–casein*, *γ-casein*, *κ-casein, WDNM1*, α–lactalbumin, *lactoferrin,* and *WAP* normalized to β-actin for 10 wk nulliparous and Days 6, 10, 14 and 18 of pregnancy. (B). Line graphs of normalized raw expression levels for milk protein gene expression throughout pregnancy. (C). Bar graphs of the rate of increase for casein expression throughout pregnancy. Expression values for each stage of preganancy are compared to the previous time point to indicate the fold increase in expression. *β-casein*, *γ-casein* and WDNM1 have their greatest increase in expression by D6. *α-lactalbumin*, *lactoferrin*, *κ-casein*, and *WAP* are up-regulated at D10 or D14 of pregnancy, while ε–casein expression is induced very late in pregnancy, D18. A similar analysis is provided for *GAPDH* and *Cytokeratin 18*.

Figure 5. *In Situ* Hybridization of Early, Intermediate and Late Differentiation Markers. Spatial analysis of *β-casein*, *WAP* and *ε-casein* expression in representative sections of nulliparity and the three trimesters of pregnancy. *β-casein* induction at D7 is representative of early markers of differentiation whereas *WAP* and *ε-casein* reflect intermediate and late differentiation events, respectively. Magnification 300x.

Figure 6. *Lactoferrin* Expression is Independent of Reproductive Variables. (A). Strain Independence. *Lactoferrin* expression is consistently elevated in parous samples independent of murine strain. (B). Regression Independence. Parity-specific expression of *lactoferrin* is maintained in both short and long regression. (C). Age at Mating Independence. Parity-induced differentiation as detected by *lactoferrin* expression is not sensitive to age at mating.

Figure 7. Early Markers of Differentiation are Sensitive to Age at Mating. Bar graphs for the expression of *β-casein, ε-casein, γ-casein, κ-casein, WDNM1, α-lactalbumin, lactoferrin,* and *WAP* normalized to *β-actin*. Membranes consist of total RNA from 15-30 nulliparous and parous animals. Parous animals are mated at 25 weeks of age and allowed 4 weeks of post-lactational involution. Early markers of differentiation no longer demonstrate parity-specific expression.

A.


Normalized Milk Protein Gene Expression
15
10
5
0
Virgin
Parous
β–casein
γ–casein
κ-casein
Lactoferrin

B.

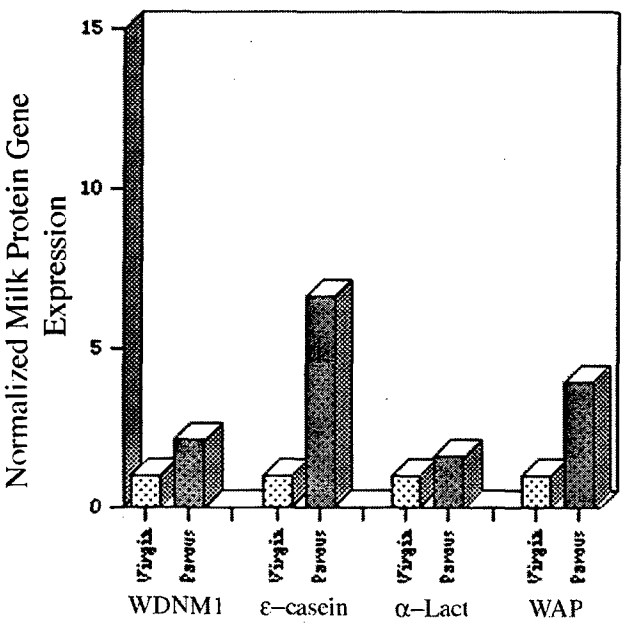

Normalized Milk Protein Gene Expression
15
10
5
0
Virgin
Parous
WDNM1
ε–casein
α–Lact
WAP

Fig. 1

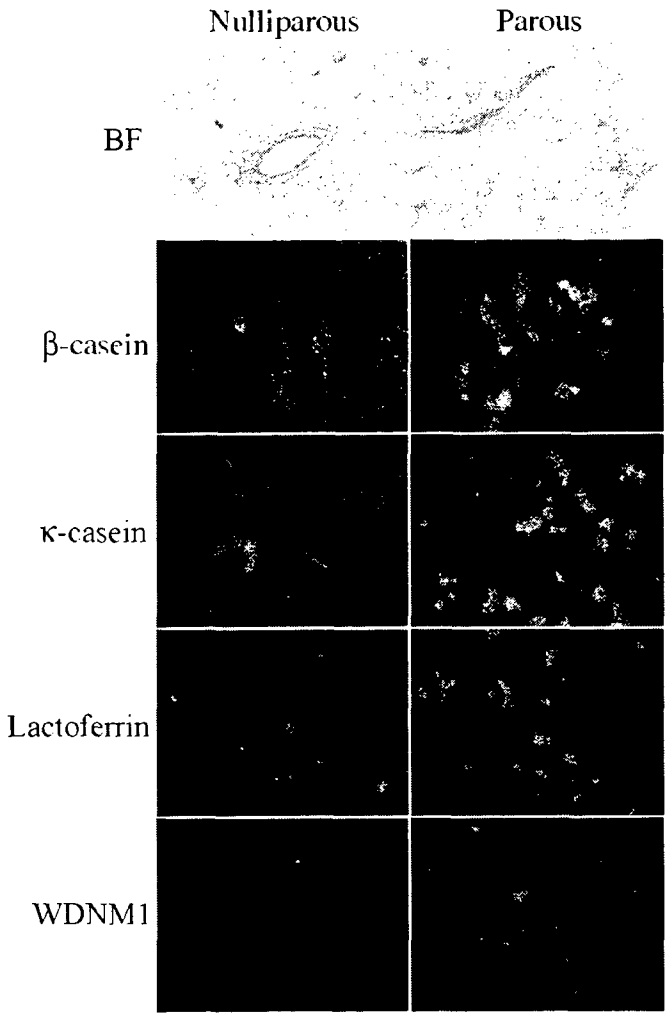
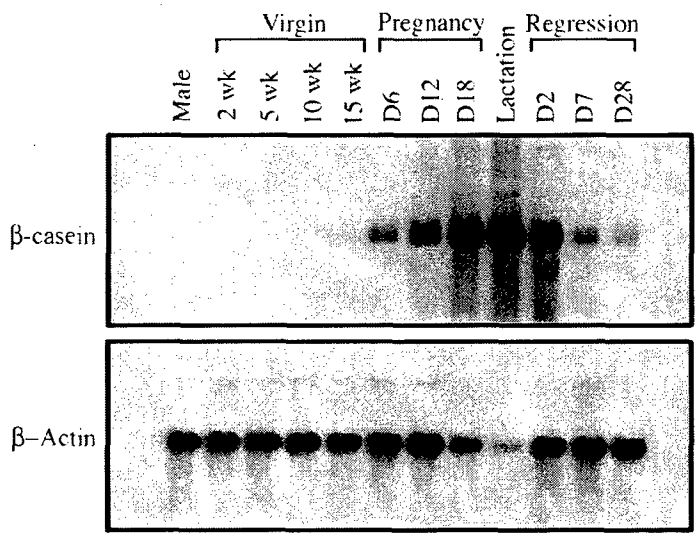


Fig. 2

A.


Virgin
Pregnancy
Regression
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
β-casein
β–Actin

B.

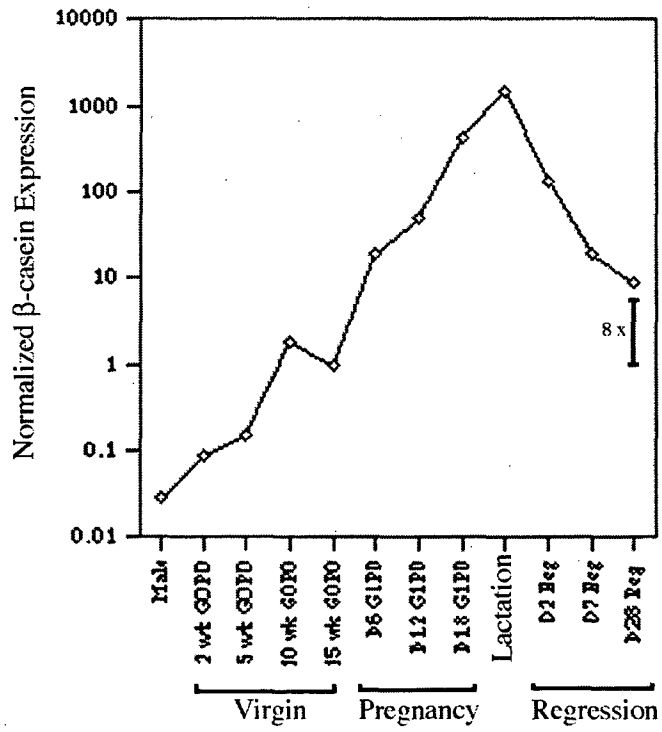

Normalized β-casein Expression
10000
1000
100
10
1
0.1
0.01
8 x
Male
2 wk GDPO
5 wk GDPO
10 wk GDPO
15 wk GDPO
D6 GIPO
D12 GIPO
D18 GIPO
Lactation
D2 Reg
D7 Reg
D28 Reg
Virgin
Pregnancy
Regression

Fig. 3

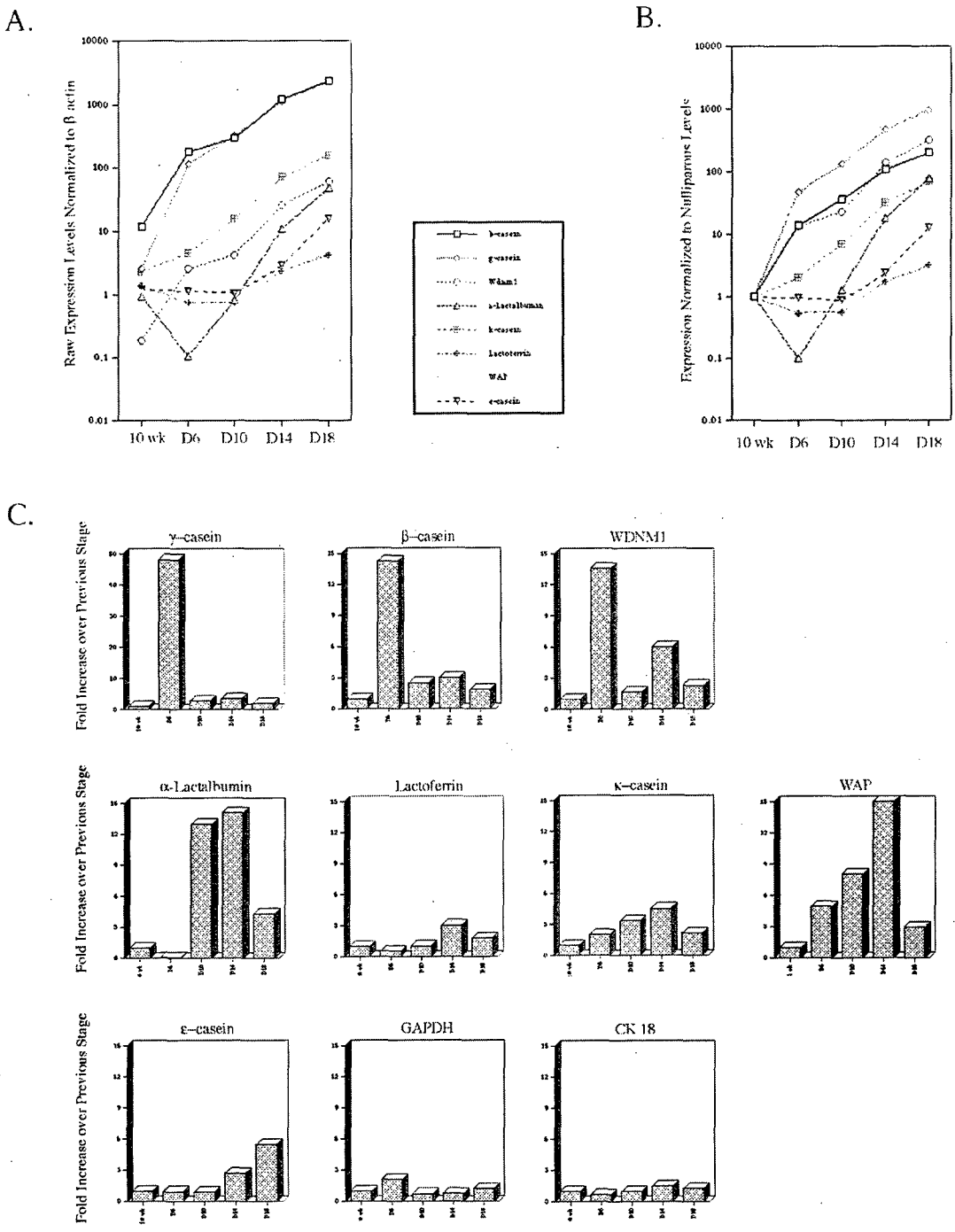

A.
B.
C.
Raw Expression Levels Normalized to β actin
Expression Normalized to Nulliparous Levels
10000
1000
100
10
1
0.1
0.01
10 wk
D6
D10
D14
D18
γ–casein
β–casein
WDNM1
α-Lactalbumin
Lactoferrin
κ–casein
WAP
ε–casein
GAPDH
CK 18
Fold Increase over Previous Stage

Fig. 4

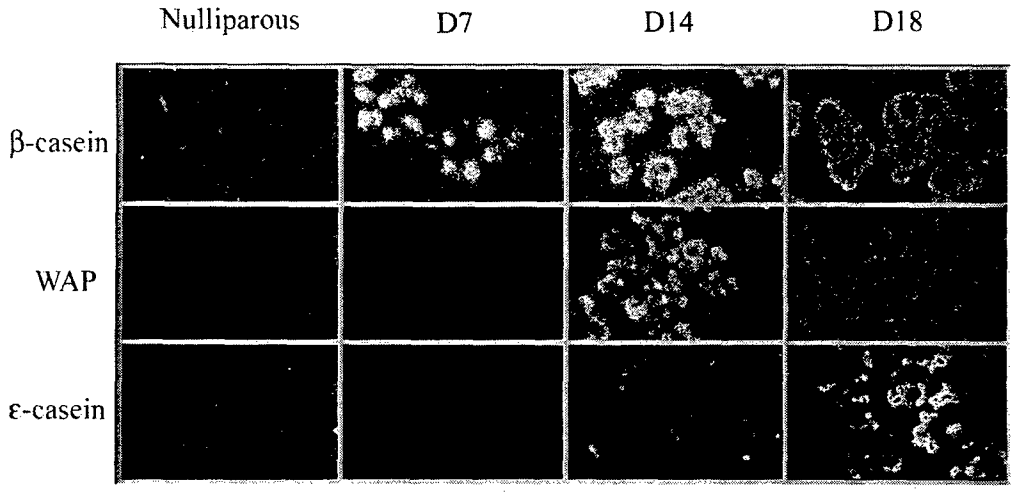
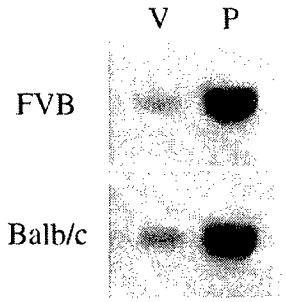


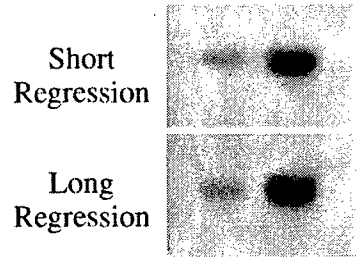
Fig. 5

A.



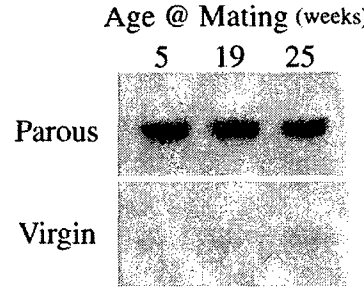
Strain Independence

B.



Regression Independence

C.



Age of First Full Term Pregnancy

Fig. 6

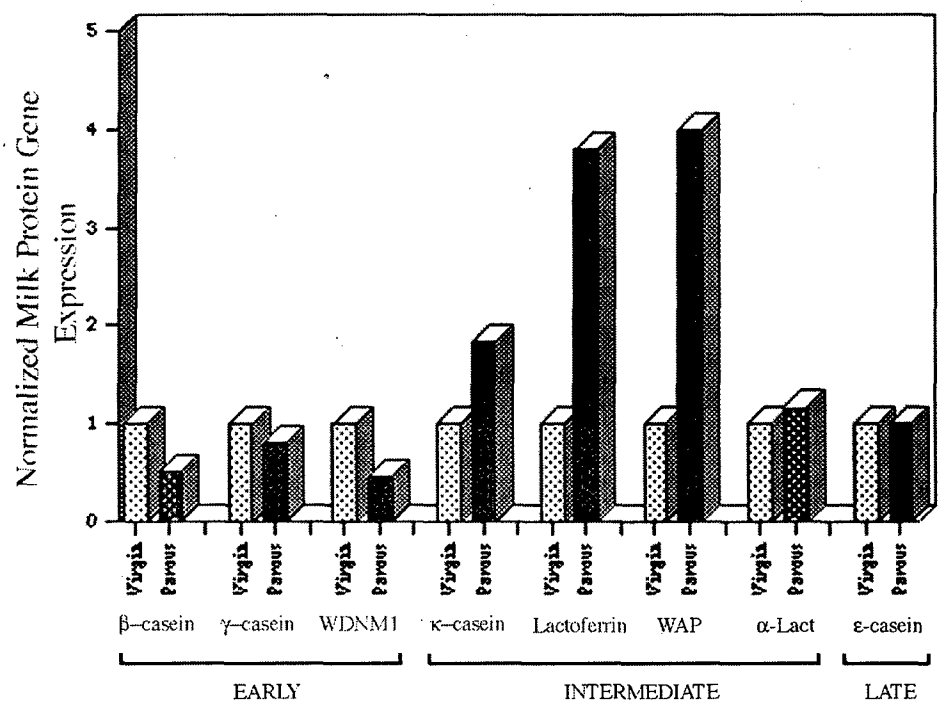

Normalized Milk Protein Gene Expression
5
4
3
2
1
0
β–casein
γ–casein
WDNM1
κ–casein
Lactoferrin
WAP
α-Lact
ε-casein
EARLY
INTERMEDIATE
LATE

Fig. 7

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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